UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

PAPEL DE LOS MICRORNAS EN LA ACTIVIDAD TERAPÉUTICA DE LAS CÉLULAS MADRE MESENQUIMALES HUMANAS

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PAPEL DE LOS MICRORNAS EN LA ACTIVIDAD TERAPÉUTICA DE LAS CÉLULAS MADRE MESENQUIMALES HUMANAS.

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INFORMAN

Que el presente trabajo titulado "Papel de los microRNAs en la actividad terapéutica de las células madre mesenquimales humanas" ha sido realizado por María Tomé Pizarro bajo nuestra dirección, en el Departamento de Desarrollo y Reparación Cardiovascular, de la Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), y lo consideramos adecuado para ser defendido como tesis doctoral.

En Madrid a 21 de Marzo de 2014

Manuel Ángel González de la Peña Antonio Bernad Miana

A mi hermano, César

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SUMARIO / SUMMARY

El remarcable potencial de las células madre mesenquimales humanas (hMSCs) para el tratamiento de diferentes patologías ha sido demostrado por numerosos estudios. Este potencial terapéutico de las hMSCs se debe a su capacidad reparadora y su habilidad de regular el sistema inmune. Sin embargo, a pesar del amplio conocimiento de las propiedades de las hMSCs, poco se sabe sobre los mecanismos encargados de regularlas.

Desde hace relativamente poco tiempo, los microRNAs (miRNAs) han emergido como potentes reguladores de las hMSCs, siendo capaces de controlar la expresión de cientos de genes de manera simultánea. En este trabajo hemos identificado el miR-335 como un protagonista indispensable en la regulación funcional de las hMSCs, siendo capaz de controlar la transición entre el estado inactivo/reposo y el estado activo/reparador de estas células. Numerosas señales encargadas de mediar la activación de las hMSCs son capaces de disminuir los niveles de miR-335, permitiendo con ello la adquisición de su fenotipo reparador.

Nuestros resultados también demuestran que la expresión de miR-335 se correlaciona positivamente con el grado de envejecimiento/senescencia en hMSCs, y su expresión exógena promueve la adquisición de un fenotipo senescente en estas células, todo ello asociado a la represión de la activación del factor de transcripción AP-1. La represión de AP-1 disminuye la capacidad de respuesta de las hMSCs ante numerosos estímulos, resultando en la supresión de sus propiedades terapeúticas (proliferación, migración, diferenciación e inmunoregulación), y manteniendo a las células en un estado de inactivación. Además, hemos podido comprobar que esta represión de AP-1 es el principal mecanismo molecular que suprime las capacidades de las hMSCs senescentes, pudiendo ser considerado un mecanismo global de represión funcional en condiciones de senescencia en dichas células.

En conclusión, hemos encontrado que miR-335 funciona como supresor de la actividad de las hMSCs, sugiriendo que las aproximaciones capaces de disminuir los niveles de este miRNA podrían ser prometedoras para aumentar la funcionalidad de las hMSCs usadas en clínica.

The remarkable potential of human mesenchymal stem cells (hMSCs) for the treatment of different diseases has been demonstrated in numerous studies. The therapeutic potential of hMSCs is due to its reparative capacity and its ability to regulate the immune system. However, despite the extensive knowledge of hMSCs properties, little is known about the mechanisms involved in their regulation.

Since relatively recently, microRNAs (miRNAs) have emerged as potent regulators of hMSCs, being able to control the expression of hundreds of genes simultaneously. In this work we have identified miR-335 as an essential player in the functional regulation of hMSCs, being able to control the transition between the inactive/resting state and active/reparative state of these cells. Numerous signals responsible for hMSCs activation are able to decrease the levels of miR-335, allowing the acquisition of its reparative phenotype.

Our results also demonstrate that the expression of miR -335 is positively correlated with the degree of aging/senescence in hMSCs, and its exogenous expression promotes the acquisition of a senescent phenotype in these cells, all associated with the suppression of the activation of the AP-1 transcription factor. Repression of AP-1 decreases the responsiveness of hMSCs to numerous stimuli, resulting in the removal of their therapeutic properties (proliferation, migration, differentiation and immune regulation), maintaining the cells in a state of inactivation. Moreover, we have found that AP-1 repression is the major molecular mechanism that suppresses the capabilities of senescent hMSCs, and can be considered as a global mechanism of the functional repression in the senescence condition of these cells.

In conclusion, we found that miR-335 works as a suppressor of hMSCs activity, suggesting that approaches capable of reducing the levels of this miRNA might be promising to increase the functionality of the hMSCs used in the clinic.

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ABREVIATURAS

3'UTR	Región no traducida 3´ (3´ untranslated region).
ACM	Células madre mesenquimales humanas de cartílago articular (Human articular cartilague mesenchymal stem cells).
ACTA2/ αSMA	Alfa 2 actina de músculo liso de aorta (Actin, alpha 2, smooth muscle, aorta).
AKAP9	Proteína de anclaje 9 (Anchor protein 9).
AKT	Proteína quinasa B (Protein kinase B).
ANGPT1	Angiopoyetina (Angiopoietin).
AP-1	Proteína activadora 1 (Activator protein 1).
ATF3	Factor de transcripción activador 3 (Activating transcription factor 3).
BGLAP/OC	Osteocalcina (Osteocalcin; Bone gamma-carboxyglutamate acid-containing protein).
BMP2	Proteína morfogenética ósea 2 (Bone morphogenetic protein 2).
BSP	Sialoproteína de hueso (Bone sialoprotein).
CCL11/EOTAXIN	Quimioquina CC ligando 11; Eotaxin (CC chemokine ligand 11; Eotaxin).
CCL13/MCP-4	Quimioquina CC ligando 13; Proteína quimiotáctica de monocitos 4 (CC chemokine ligand 13; Monocyte chemotactic protein-4).
CCL2/MCP-1	Quimioquina CC ligando 2; Proteína quimiotáctica de monocitos 1 (CC chemokine ligand 2; Monocyte chemotactic protein-1).
CCL3/(MIP-1α)	Quimioquina CC ligando 3; Proteína inflamatoria de macrófagos 1α (CC chemokine ligand 3; Macrophage inflammatory protein-1α).
CCL5/RANTES	Quimioquina CC ligando 5; Factor de regulación tras la activación normal de los linfocitos (CC chemokine ligand 5; Regulated on activation, normal T cell expressed and secreted).
CCND1	Ciclina D1 (Cyclin D1).
CCR5/CD195	Quimioquina CC receptor 5 (CC chemokine receptor 5).
CD105/ Eng	Endoglina (Endoglin).
CD11a	Cadena alfa L de integrina (Integrin, alpha L).
CD11b/ITGAM	Cadena alfa M de integrina (Integrin alpha M).
CD14	Molécula CD14 (CD14 molecule).
CD18	Integrina beta 2 (Integrin, beta 2).
CD19	Antígeno CD19 (CD19 antigen).
CD200	Antígeno CD200 (CD200 antigen).
CD24	Molécula CD24 (CD24 molecule).
CD274/PD-L1	Molécula CD274; Ligando de muerte programada (CD274 molecule; Programmed death ligand 1).
CD34	Molécula CD34 (CD34 molecule).
CD4+	Linfocitos T colaboradores (Mature T helper cells).
CD40	Molécula CD40; miembro 5 de la superfamilia de receptores de TNF (CD40 molecule, TNF receptor superfamily member 5).
CD45-PTPRC	Receptor tipo C; Proteína tirosina fosfatasa (Protein tyrosine phosphatase, receptor type, C).
CD79a	Molécula CD79a (CD79a molecule).
CD8+	Linfocitos T citotóxicos (Cytotoxic T cells).
CD80	Molécula CD80 (CD80 molecule).
CD86	Molécula CD86 (CD86 molecule).
CD90	Sinónimo de Thy1.
CDKN1A/p21	Inhibidor dependiente de quinasa 1A (Cyclin-dependent kinase inhibitor 1A).
CDKN2A/p16	Inhibidor dependiente de quinasa 2A (Cyclin-dependent kinase inhibitor 2A).
CM	Medio condicionado (Conditioned medium).
CM-Mϕ	Medio condicionado de macrófagos activados (Conditioned medium from activated macrophages).
COX2	Ciclooxigenasa 2, sinónimo de PTGS2.

CPD	Duplicaciones celulares acumuladas (Cumulative population doubling).
CSF3	Factor estimulante de colonias de granulocitos (Granulocyte colony-stimulating factor).
CX3CL1	Quimioquina CX3C ligando 1 (CX3C chemokine ligand 1).
CXCL1	Quimioquina CXC ligando 1 (CXC chemokine ligand 1).
CXCL10	Quimioquina CXC ligando 10 (CXC chemokine ligand 10).
CXCL12/SDF1	Quimioquina CXC ligando 12; Factor 1 derivado de células estromales (CXC chemokine ligand 12; Stromal cell-derived factor 1).
CXCL2	Quimioquina CXC ligando 2 (CXC chemokine ligand 2).
CXCL3	Quimioquina CXC ligando 3 (CXC chemokine ligand 3).
CXCR3	Quimioquina CXC receptor 3 (CXC chemokine receptor 3).
CXCR4	Receptor de quimioquina CXC tipo 4 (Chemokine CXC receptor 4).
Daam1	Activador desarreglado asociado a la morfogénesis-1 (Dishevelled associated activator of morphogenesis 1).
daf-12	Proteína DAF-12 (Protein DAF-12).
DHE	Dihidroetidio (Dihydroethidium).
Dicer	Ribonucleasa tipo III Dicer 1 (Dicer 1, ribonuclease type III).
DKK-1	Dickkopf homólogo 1 (Dickkopf homolog 1).
DMSO	Dimetil-sulfóxido (Dimethyl Sulfoxide).
EDN1	Endotelina 1 (Endothelin 1)
EdU	5-etinil-2-deoxiuridina (5-ethynyl-2-deoxyuridine).
EGF	Factor de crecimiento epidermal (Epidermal growth factor).
ERK	Quinasa reguladora de señales extracelulares (Extracellular signal-regulated kinases).
ESC	Células madre embrionarias (Embryonic stem cells).
FBS	Suero fetal bovino (Fetal bovine serum).
FGF	Factor de crecimiento de fibroblastos (Fibroblast growth factor).
FITC	Isotiocianato de fluoresceína (Fluorescein Isothiocyanate).
FOS	Oncogén FBJ homólogo de osteosarcoma murino (FBJ murine osteosarcoma viral oncogene homolog).
Fox-2	Proteína de unión al RNA, homólogo 2 de fox-1 (RNA binding protein, fox-1 homolog 2).
FPR1/fMLP	Receptor del péptido formil (Formyl peptide receptor 1).
GFP	Proteína verde fluorescente (Green fluorescen protein)
GM-CSF	Factor estimulante de colonias de granulocitos y macrófagos (Granulocyte-macrophage colony-stimulating factor).
GMP	Buenas prácticas de fabricación (Good manufacturing practices)
GO	Ontología génica (Gene ontology).
H19	Transcrito maternalmente imprintado H19 (H19, imprinted maternally expressed transcrip).
HGF	Factor de crecimiento de hepatocitos, hepapoyetina A (Hepatocyte growth factor; Hepapoietin A).
HLA-DR	Complejo mayor de histocompatibilidad clase II, DR beta 1 (Major histocompatibility complex, class II, DR beta 1).
HLA-G1	Complejo mayor de histocompatibilidad clase I, G (Major histocompatibility complex, class I, G).
hMG1	Grupo de alta movilidad (High mobility group box 1).
hMSCs	Células madre mesenquimales humanas (Human mesenchymal stem cells).
hMSCs-MO	Células madre mesenquimales humanas de médula ósea (Bone marrow human mesenchymal stem cells).
HO1	Hemooxigenasa 1 (Heme oxygenase 1).
HSC	Células madre hematopoyéticas (Hematopoyetic stem cells).
hTERT	Subunidad reverso-transcriptasa de la telomerasa (human telomerase reverse transcriptase).
ICAM1	Molécula de adhesión intercelular (Intercellular adhesion molecule 1).

IDO	Indoleamina 2,3-dioxygenasa (Indoleamine 2,3-dioxygenase).
IFNβ	Interferón beta-1a (Interferon beta-1a).
IFNγ	Interferón gamma (Interferon, gamma).
IGF1	Factor de crecimiento insulínico 1 (Insulin-like Growth Factor 1).
IGF2	Factor de crecimiento insulínico 2 (Insulin-like Growth Factor 2).
IL12B/ IL12P40	Subunidad beta de la interleuquina 12 (Subunit beta of interleukin 12; Natural killer cell stimulatory factor 2; Cytotoxic lymphocyte maturation factor 2, p40).
IL15	Interleuquina 15 (Interleukin 15).
IL17F	Interleuquina 17F (Interleukin 17F).
IL1RA	Antagonista del receptor de interleuquina-1 (Interleukin-1 receptor antagonist).
IL2	Interleuquina 2 (Interleukin 2).
IL4	Interleuquina 4 (Interleukin 4).
IL6	Interleuquina 6 (Interleukin 6).
IL8	Interleuquina 8 (Interleukin 8).
ILa/IL1A	Interleuquina 1 alfa (Interleukin 1, alpha).
ILb/IL1B	Interleuquina 1 beta (Interleukin 1, beta).
iNOS	Óxido nítrico sintasa inducible (Inducible nitric oxide synthase).
ISCT	Sociedad internacional de terapia celular (International Society for Cell Therapy).
JNK	Quinasa N-terminal de c-JUN (c-Jun N-terminal kinase JNK).
JUN	Protooncogen JUN (Jun proto-oncogene).
KLF4	Factor tipo Kruppel 4 (Kruppel-like factor 4).
LEP	Leptina (Leptin).
let-7	Precursor de miRNA let-7 (let-7 microRNA precursor).
LIF	Factor inhibidor de leucemia (Leukemia inhibitory factor).
lin-14	Proteína LIN-14 (Protein LIN-14).
lin-28	Proteína LIN-28 (Protein LIN-28).
lin-41	Proteína LIN-41 (Protein LIN-41).
lin-42	Proteína LIN-42 (Protein LIN-42).
LPS	Lipopolisacárido (Lipopolysaccharide).
MAF	Homólogo del oncogén del fibrosarcoma musculoaponeurótico de ave (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog).
MAPK	Proteína quinasa activada por mitógenos (Mitogen-activated protein kinase).
Mapk14/p38	Proteína quinasa activada por mitógenos 14 (Mitogen-activated protein kinase 14).
mESC	Células madre embrionarias de ratón (Mouse embryonic stem cells).
MEST	Transcrito específico de mesodermo (Mesoderm specific transcript).
MHC-I/HLA	Complejo de histocompatibilidad tipo I (Major histocompatibility complex type I).
MHC-II	Complejo de histocompatibilidad tipo II (Major histocompatibility complex type II).
miRNA	microRNA (microRNA).
MKK7	MAP quinasa quinasa tipo 7 (MAP kinase kinase 7).
MMP	Metaloproteinasa de matriz (Matrix metalloproteinase).
MMP1	Metaloproteinasa de matriz 1 (Matrix metallopeptidase 1).
MMP2	Metaloproteinasa de matriz 2 (Matrix metallopeptidase 2).
MMP9	Metaloproteinasa de matriz 9 (Matrix metallopeptidase 9).
mMSCs	Células madre mesenquimales de ratón (Mouse mesenchymal stem cells).
MO	Médula ósea (Bone marrow).
MRC1/CD206	Receptor de manosa C tipo 1 (Mannose receptor, C type 1).
MSCs	Células madre mesenquimales (Mesenchymal stem cells).

MSCs-miR-335	MSCs transducidas con la construcción lentiviral que codifica para miR-335 (MSCs transduced with the lentiviral construction encoding miR-335).
MSCs-control	MSCs transducidas con la construcción lentiviral que codifica para el shRNA control negativo (MSCs transduced with the lentiviral construction encoding the negative control shRNA).
MSCs-MO	Células madre mesenquimales de médula ósea (Bone marrow mesenchymal stem cells).
NGF	Factor de crecimiento nervioso (Nerve growth factor).
NK	Células NK (Natural killer cells).
NMA	Análisis morfométrico nuclear (Nuclear morphometric analysis).
NT5E/ CD73	5´nucleotidasa (5'-nucleotidase, ecto).
ob/ob	Ratón obeso (Obese mouse).
OSM	Oncostatina M (Oncostatin M).
p-	Forma fosforilada (Phosphorilated form).
p16	Sinónimo de CDKN2A.
p21	Sinónimo de CDKN1A.
p53	Proteína tumoral P53 (Tumor protein P53).
PBMC	Célula mononuclear de sangre periférica (Peripheral blood mononucleated cell).
PBS	Tampón salino de fosfato (Phosphate buffered saline).
PDGF	Factor de crecimiento derivado de plaquetas (Platelet-derived growth factor beta).
PDGFBB	Factor de crecimiento derivado de plaquetas beta (Platelet-derived growth factor beta).
Peg1	Gen 1 expresado paternalmente (Paternally-Expressed Gene 1).
PEG2	Prostaglandina E2 (Prostaglandin E2).
PMA	Forbol 12-miristato 13-acetato (Phorbol 12-myristate 13-acetate).
PRKD1	Proteína quinasa D1 (Protein kinase D1).
PTGS2/COX2	Prostaglandina-endoperóxido sintasa 2 (Prostaglandin-endoperoxide synthase 2).
qRT-PCR	Reacción en cadena de la polimerasa con transcriptasa reversa en tiempo real (Real time reverse transcription polymerase chain reaction).
RB1/pRB	Proteína del retinoblastoma (Retinoblastoma protein).
Fox-2	Proteína de unión al RNA, homólogo 2 de fox-1 (RNA binding protein, fox-1 homolog 2).
RISC	Complejo de silenciamiento inducido por RNA (RNA-induced silencing complex).
ROCK1	Proteína quinasa 1 asociada a Rho (Rho-associated, coiled-coil containing protein kinase 1).
ROS	Especies Reactivas del Oxígeno (Reactive Oxygen Species).
rSEN	Senescencia inducida por replicación (Replication induced senescence).
qRT-PCR	Reacción en cadena de la polimerasa con transcriptasa reversa en tiempo real (Real time reverse transcription polymerase chain reaction).
RUNX2	Factor de transcripción 2 relacionado con Runt (Runt related transcription factor 2).
SA-β-gal	Galactosidasa beta asociada a senescencia (Senescence associated beta galactosidase).
SASP	Fenotipo secretor asociado a senescencia (Senescence-associated secretory phenotype).
SCIN	Escinderina (Scinderin).
SELP/selectina-P	Selectina P (Selectin P).
SIRT1	Sirtuina 1 (Sirtuin 1).
SOD2	Superóxido dismutasa 2 (Superoxide dismutase 2).
SOX17	SRY (región Y determinante del sexo) tipo 17 (SRY (sex determining region Y)-box 17).

SOX4	SRY (región Y determinante del sexo) tipo 4 (SRY (sex determining region Y)-box 4).
SOX9	SRY (región Y determinante del sexo) tipo 9 (SRY (sex determining region Y)-box 9).
SRF	Factor de respuesta al suero (Serum response factor).
TAMs	Macrófagos asociados a tumores (Tumor-associated macrophages).
TERT	Subunidad transcriptasa reversa de telomerasa (Telomerase reverse transcriptase).
TGFα	Factor de crecimiento transformante alfa (Transforming growth factor, alpha).
TGFβ	Factor de crecimiento transformante beta (Transforming growth factor, beta 1).
Th1	Linfocitos T colaboradores de tipo 1 (Type 1 helper T cells).
THY1/ CD90	Antígeno de superficie celular Thy-1 (Thy-1 cell surface antigen).
TLR4	Receptor de tipo Toll 4 (Toll-like receptor 4).
TLRs	Receptores de tipo Toll (Toll-like receptors).
TNC	Tenascina C (Tenascin C).
TNFα	Factor de necrosis tumoral (Tumor necrosis factor).
TNFAIP6	Proteína inducible del gen del factor de necrosis tumoral (Tumor necrosis factor-inducible gene 6 protein).
U2OS	Línea celular de osteosarcoma humano (Human osteosarcoma cell line).
UBE2F	Enzima conjugadora de ubiquitina E2F (Ubiquitin-conjugating enzyme E2F).
VCAM1	Molécula de adhesión celular 1 (Vascular cell adhesion molecule 1).
VEGFA	Factor de crecimiento vascular endotelial A (vascular endothelial growth factor A).
VLA-4	Antígeno 4 muy tardío (Very late antigen-4).
VTCN1/B7-H4	Inhibidor de la activación 1 conteniendo el dominio V-set (V-set domain containing T cell activation inhibitor 1).
Wnt/β-catenina	Ruta wnt/beta-catenina (Wnt/beta-catenin pathway).
WNT3a	Miembro 3A de la familia del sitio de integración MMTV de tipo wingless (WNT) (Wingless-type MMTV integration site family, member 3A).
ZMPSTE24	Metaloproteinasa de zinc STE24 (Zinc metallopeptidase STE24).

INTRODUCCIÓN

"La gran promesa terapéutica". Éste ha sido el término que muchos profesionales de la biomedicina han usado, en esta última década, para describir a las células madre mesenquimales (MSCs), debido al gran entusiasmo que ha generado su eficacia en el tratamiento de una amplia variedad de enfermedades. A día de hoy, las MSCs humanas (hMSCs) han sido evaluadas en multitud de ensayos clínicos, mostrando la seguridad de su uso, así como su eficacia terapéutica. La utilidad de las hMSCs en el tratamiento de diversas patologías se debe a sus propiedades biológicas, las cuales son capaces de promover la reparación de los tejidos y modular la respuesta inmune. Estas propiedades permiten a las hMSCs la reducción del daño tisular, así como el mantenimiento funcional y estructural de los diferentes órganos, procesos que en su conjunto se conocen como *homeostasis tisular*.

Mientras numerosos grupos se centran en la investigación de nuevas aplicaciones clínicas de las MSCs, otros muchos profundizan en el estudio de sus propiedades biológicas para la mejora de su eficiencia terapéutica. Una de las principales limitaciones de estas células es que sus propiedades terapéuticas van disminuyendo a medida que envejecen. Este proceso de envejecimiento de las MSCs se produce tanto *in vivo* (en el donante del que proceden), como durante su expansión *ex vivo*, proceso necesario para su posterior uso en clínica.

Dentro de los mecanismos reguladores de la biología de las MSCs, los microRNAs (miRNAs) han emergido como los principales protagonistas, participando en la modulación de numerosas rutas centrales. En este trabajo nos hemos centrado en la identificación, regulación y caracterización funcional de miR-335, como un protagonista indispensable capaz de controlar las propiedades terapéuticas de las MSCs humanas.

1. CÉLULAS MADRE.

Definición.

Las células madre, también llamadas células troncales, son las células responsables de la formación y regeneración de los tejidos durante toda la vida del organismo, desde el estado embrionario al adulto. Estas células están dotadas simultáneamente de la capacidad de autorrenovación y del potencial de diferenciación[1].

La autorrenovación se define como la capacidad de dividirse, de forma que una (división simétrica) o ambas (división asimétrica) células hijas permanecen indiferenciadas,

manteniendo la habilidad de dar lugar a nuevas células madre con características similares a la célula de origen. El **potencial de diferenciación** es la capacidad de originar diferentes linajes celulares con características y funciones especializadas bajo las condiciones o señales apropiadas del microambiente.

Clasificación.

Las células madre se pueden clasificar principalmente según dos criterios: su potencial de diferenciación y su origen.

- Según su **potencial de diferenciación** se pueden dividir en: **totipotentes,** capaces de generar un organismo completo (tejido embrionario y extraembrionario); **pluripotentes**, células que pueden dar origen a progenitores de cualquiera de las tres capas germinales embrionarias (mesodermo, endodermo y ectodermo); **multipotentes**, aquellas capaces de originar precursores relacionados solamente con una de las tres capas embrionarias; y **unipotentes,** células muy comprometidas y con un potencial de diferenciación muy limitado que se diferencian hacia un único tipo celular.

- **Según su origen,** las células madre se clasifican de acuerdo a la fase de desarrollo en la que están presentes, dividiéndose en: embrionarias, fetales o adultas. Las **células madre embrionarias ó ESCs** (del inglés, *Embryonic Stem Cells*) proceden de embriones antes de su implantación en el útero, y son células pluripotentes. Fueron aisladas por primera vez en 1988[2]. **Las células madre fetales** son células multipotentes que se encuentran en los órganos del feto en desarrollo, y poseen características similares a sus homólogas en tejidos adultos[3]. En el caso de las células madre presentes en el organismo adulto se denominan genéricamente **células madre adultas** o células madre somáticas. Éstas son células relativamente indiferenciadas, y pueden originar diferentes tipos celulares más especializados. En la actualidad, la lista de células madre adultas caracterizadas es muy amplia, pero de entre todas ellas, las células madre mesenquimales son unas de las que más atención han concentrado a lo largo de los últimos años.

Células madre mesenquimales.

Las células madre mesenquimales o MSCs (del inglés *Mesenchymal Stem Cells* o *Mesenchymal Stromal Cells*) son células madre adultas multipotentes, identificadas por primera vez en 1966 como células de médula ósea[4]. Las MSCs son capaces de formar colonias y de diferenciar a otros tipos celulares mesodérmicos como hueso y cartílago, tanto *in vitro* como después de su transferencia *in vivo*[5]. Como resultado de esta capacidad

de autorrenovación y diferenciación, estas células fueron consideradas células madre por Caplan, y llamadas MSCs[6]. Posteriormente, se les atribuyó la capacidad de diferenciar a otros tipos celulares de linaje endodérmico y ectodérmico[7-9], y más recientemente se descubrió su capacidad de regular el sistema inmune[10].

Fuentes de MSCs.

En cuanto a su localización, se puede decir que estas células se encuentran formando parte del estroma de virtualmente todos los órganos[11]. De entre todos ellos, el tejido adiposo presenta un interés especial como fuente de MSCs, pues se trata de un tejido abundante y de fácil acceso por liposucción, del que pueden obtenerse con gran eficacia una gran cantidad de MSCs. Sin embargo, la mayoría de los estudios se han llevado a cabo con MSCs procedentes de médula ósea (MSCs-MO), siendo hasta el momento las más estudiadas. Las MSCs de la médula ósea humana (hMSCs-MO) representan únicamente el 0,01-0,001% de las células nucleadas de dicho tejido.

Caracterización de las MSCs.

Con el fin de establecer unas pautas comunes para la caracterización de las MSCs, la ISCT (del inglés *International Society for Cell Therapy)* recomendó en el año 2006 un criterio mínimo estándar que consiste en: (1) crecimiento en adherencia a las superficies plásticas utilizadas para su cultivo; (2) expresión de las moléculas de superficie CD73 CD90 y CD105, y la ausencia de marcadores de monocitos y macrófagos (CD11b y CD14), de progenitores hematopoyéticos y marcadores de células endoteliales (CD34), marcador lecocitario (CD45), marcador de células B (CD19 ó CD79a) y HLA-DR; y (3) capacidad de diferenciación hacia tejido óseo, cartilaginoso y adiposo *in vitro* **(Figura 1)**.

Otra característica muy importante de las MSCs es su denominada condición **"inmunoprivilegiada"**, es decir, son capaces de evadir el rechazo del sistema inmune durante largos periodos de tiempo[12]. Esta capacidad se debe a que expresan niveles muy bajos del complejo de histocompatibilidad tipo I (MHC-I) y no expresan el tipo II (MHC-II) ni las moléculas coestimulatorias CD40, CD80 y CD86. Aunque los niveles de los antígenos de MHC-I son muy bajos, pueden activar a las células T, pero la ausencia de moléculas coestimulatorias hace que no se puedan iniciar las señales secundarias. Esto permite reducir el rechazo en trasplantes alogénicos, lo cual constituye una gran ventaja en el tratamiento de enfermedades donde el daño o pérdida celular imposibilita el uso de las células del propio paciente[13,].

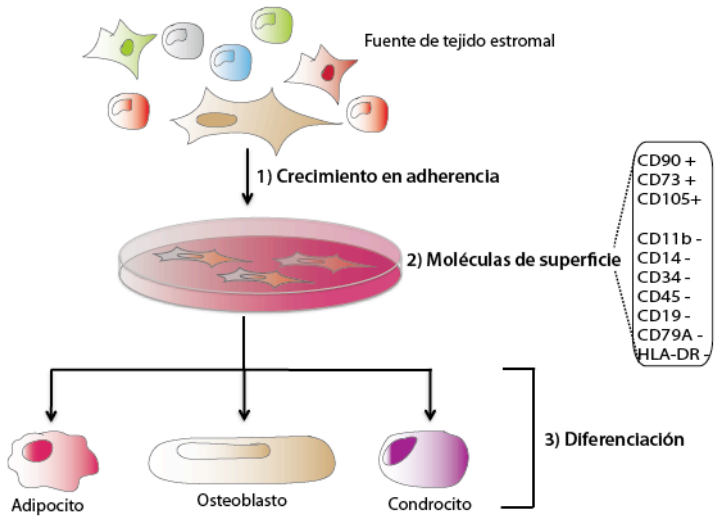

Figura 1. Criterio para la definición de las MSCs. Las MSCs son distinguidas de otras células gracias a su adherencia a las placas de cultivo y a que son capaces de diferenciar *in vitro* a osteoblastos, condrocitos y adipocitos. Además, las MSCs se definen por la expresión de los marcadores de superficie CD73, CD90 y CD105, y la ausencia de CD34, CD45, CD14, CD11b, CD79a, y HLA-DR. Adaptado de Le Blanc y Mougiakakos[14].

Nicho celular.

El concepto de "nicho" celular fue descrito por primera vez en el año 1978 por Schofield[15]. Se define como el microambiente que permite la transferencia de señales a través de interacciones por contacto celular, factores solubles y matriz extracelular, las cuales regulan el balance de las células madre según las necesidades del organismo. El nicho, de esta manera, mantiene a las células madre en un estado quiescente o inactivo, o por el contrario promueve su activación para responder frente a diferentes estímulos, mediando el reemplazo de células muertas o disfuncionales (homeostasis), o reparando el tejido después de sufrir un daño.

Estudios recientes sugieren la naturaleza perivascular de los nichos de las MSCs, debido a que estas células, independientemente del tejido de origen, expresan alfa actina de músculo liso (αSMA), la cual se encuentra en grandes cantidades en las estructuras vasculares[16]**.** Esta localización perivascular a lo largo de todo el organismo permitiría teóricamente el acceso de las MSCs a todos los tejidos. Sin embargo, algunos otros grupos sugieren que también podrían encontrarse en tejidos avasculares como el cartílago articular[17-19].

Propiedades terapéuticas de las MSCs.

En condiciones normales, las células apoptóticas o necróticas son eliminadas por los macrófagos residentes sin causar inflamación. Por el contrario, si se produce un daño, éste es seguido por un proceso de inflamación, incluso en casos en los que no haya habido un

proceso infeccioso, lo cual permite la activación de las células del sistema inmune como células T, o células B[20, 21]. Los componentes liberados por las células necróticas, la microvasculatura dañada (formada principalmente por células endoteliales) y las células activadas del sistema inmune, promueven la liberación de varios factores proinflamatorios como TNFα, ILα, Ilβ, IFNγ[22] y una gran cantidad de factores quimioatrayentes que forman un gradiente que dirige la movilización de las MSCs al lugar del daño **(migración)**, incluso en el caso de que las MSCs no residan en el propio tejido dañado.

Por otro lado, el conjunto formado por las células del sistema inmune, las células endoteliales, y las propias moléculas proinflamatorias, crea un microambiente que activa a las MSCs y las estimula a participar en la reparación del tejido. La activación de las MSCs las induce a **proliferar y diferenciar en** los tipos celulares que han sido dañados, y a **secretar numerosos mediadores** que ayudan a disminuir el proceso de inflamación y contribuyen a la reparación del tejido.

Proceso básico de reparación tisular por MSCs.

El proceso de reparación tisular es muy complejo, ya que consta de numerosas etapas y mecanismos muy diferentes, que en su conjunto permiten la recuperación de la integridad funcional y estructural del tejido dañado **(Figura 2)**.

a) Migración.

El primer paso consiste en la movilización de las MSCs desde sus nichos a la circulación hasta alcanzar el lugar dañado. Este proceso es a su vez dividido en varias etapas:

a.1) Quimiotaxis. El hecho de que las MSCs expresen una gran variedad de receptores para quimioquinas sugiere que poseen un potencial de movilización hacia muy diversos tejidos[23]. Este potencial se encuentra bajo el control de una gran cantidad de quimioquinas y factores de crecimiento que son secretados por el tejido dañado. Así, varios estudios han demostrado la capacidad de migración de las MSCs frente a citoquinas quimioatrayentes como CCL3[24], IL8α[25], CXCL12 (SDF1; revisado en[26]), CX3CL1 y CXCL10[27]. También ha sido probada la migración de las MSCs en respuesta a diferentes factores de crecimiento, entre ellos CXCL12, PDGF y FGF, generados por los tejidos dañados[25, 28, 29]. Además, la preincubación de las MSCs con citoquinas proinflamatorias como TNFα e IFNγ, solas o en combinación, es capaz de incrementar la expresión de sus receptores, aumentando el potencial de migración de las MSCs[27].

La pareja quimioquina/receptor más estudiada en el proceso de migración de las MSCs ha sido CXCL12/CXCR4. CXCL12 y su receptor CXCR4 son, además, fundamentales para la retención en la médula, movilización y reclutamiento de células madre hematopoyéticas[30] y células progenitoras endoteliales[31]. Otro grupo demostró que CXCR4 está altamente expresado en una subpoblación de hMSCs cuya migración es dependiente de CXCL12[32]. Más recientemente, se ha visto que las rutas de AKT, ERK y p38 parecen estar implicadas en la migración mediada por CXCL12[33].

También cabe destacar que las MSCs tienen un papel muy importante en la patogénesis y progresión de tumores, puesto que éstos son sitios de alta producción de citoquinas y quimioquinas proinflamatorias, lo cual permite la migración de las MSCs hacia el ambiente tumoral[34].

a.2) Rodamiento, adhesión y transmigración endotelial. La extravasación de las MSCs a los sitios de daño requiere la activación previa del endotelio por TNFα, el cual estimula la expresión de una gran cantidad de quimioquinas y moléculas de adhesión.

- Rodamiento: Primeramente se produce el proceso de "***rolling*"** o **rodamiento,** es decir, las MSCs son capaces de moverse por el torrente sanguíneo, rodando sobre las células endoteliales de la vasculatura**.** El grupo de Ruster demostró que las hMSCs se unían a células endoteliales y rodaban sobre ellas, con una rápida extensión de podios, mediante la unión entre el ligando de selectinas CD24 (presente en la membrana de las MSCs), y la selectina-P (expresada en las células endoteliales)[35].

- **Adhesión:** Posteriormente, las MSCs son retenidas por la vasculatura del tejido, produciéndose una adhesión firme al mismo, mediada por integrinas y moléculas de adhesión. Así, la integrina VLA-4 (o el dímero de integrina beta 1 e integrina alfa 4) expresada en las MSCs es capaz de unirse con VCAM1 en las células endoteliales[35]. Otra integrina expresada por MSCs es CD11a/CD18, la cual se une a ICAM1 de las células endoteliales, contribuyendo también al proceso de adhesión[36].

- **Transmigración:** Por último, se produce la migración celular a través de las paredes de la vasculatura del endotelio, proceso conocido como transmigración. Dos estudios han examinado la trasmigración *in vitro,* usando un cocultivo de MSCs con células endoteliales[37, 38].

Ambos estudios demuestran que las MSCs sufren cambios morfológicos y que su integración a través de la capa endotelial, mediante podios de membrana, puede ser paracelular (entre las células endoteliales) o transcelular (directamente a través de las células endoteliales), es decir, mediante diapédesis a través de poros en la capa endotelial.

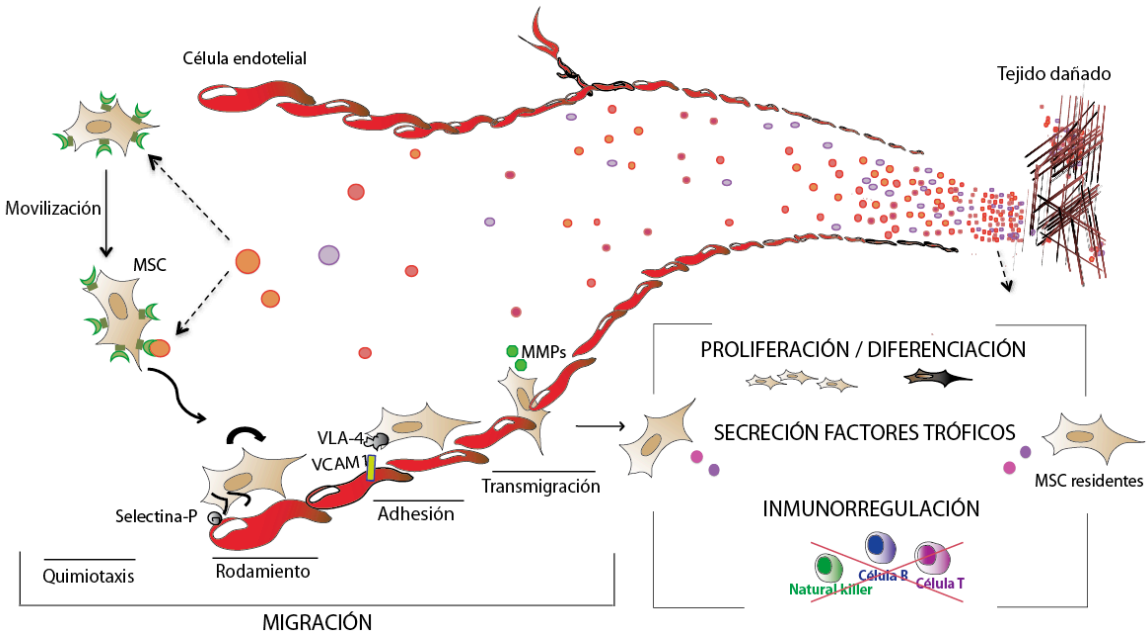

Figura 2. **Proceso básico de reparación tisular por MSCs**. El daño en un tejido produce la activación del sistema inmune, resultando en la liberación de una gran cantidad de moléculas proinflamatorias, tanto por las propias células del sistema inmune, como por las células dañadas. El gradiente de moléculas producido atrae a las MSCs desde sus nichos hasta la circulación (quimiotaxis). Posteriormente, las MSCs ruedan sobre las células endoteliales que forman la vasculatura (rodamiento), y se adhieren firmemente mediante integrinas a las células endoteliales (adhesión). Finalmente, las MSCs migran a través de la pared endotelial hasta alcanzar el tejido dañado (transmigración), donde proliferan y diferencian a los tipos celulares que han sido dañados. También secretarán al medio una serie de factores tróficos gracias a los cuales, entre otras funciones mediarán la inmunorregulación. Otras MSCs residentes en el lugar dañado también contribuyen a la reparación del tejido mediante los mismos procesos a excepción de la migración.

a.3) Invasión. Es el último paso antes de alcanzar el lugar del daño. Se caracteriza porque las MSCs atraviesan la membrana basal y la matriz extracelular del tejido dañado. En esta función juegan un papel muy importante las metaloproteasas (MMP), enzimas proteolíticas encargadas de la degradación de los componentes de la membrana basal vascular, la cual está formada por diferentes proteínas de matriz extracelular como lamininas, colágeno tipo IV y gelatina. Así, por ejemplo, se ha demostrado que las MSCs expresan MMP1, la cual degrada el principal componente de la membrana basal, el colágeno IV[39]. También se ha señalado la implicación de MMP2, una gelatinasa, en el mismo proceso[40].

b) Proliferación y diferenciación.

Una vez que las MSCs han llegado al tejido en cuestión, son capaces de proliferar y, en muchos casos, diferenciarse en los tipos celulares que han sido dañados, dando lugar a la formación de un tejido maduro y funcional[41].

Numerosos estudios *in vitro* han demostrado la multipotencialidad de las MSCs, dando una primera idea de su potencial reparador. Además, diversos estudios *in vivo* han

demostrado que las MSCs pueden disminuir el daño en el tejido y mejorar la función después del mismo en pulmón[42], riñón[43, 44], hígado[45, 46], diabetes[46, 47] e infarto de miocardio[48, 49].

Papel de los macrófagos en la regulación de la migración y diferenciación de las MSCs.

Los macrófagos son el principal componente de la respuesta inflamatoria frente al daño y, al igual que las MSCs, llevan a cabo cambios en su fenotipo según los estímulos locales del microambiente. Dichos cambios incluyen la secreción de diferentes factores que actúan directamente sobre las MSCs, regulando sus capacidades de migración y diferenciación.

- **Migración:** Varios trabajos demuestran que los macrófagos activados por diferentes estímulos, por ejemplo LPS (lipopolisacárido, un componente de la pared de bacterias Gram-), o PMA (del inglés, *phorbol myristate acetate,* un éster de forbol muy usado para activar macrófagos), exhiben un incremento en la producción de varias citoquinas, entre ellas: IL6, TNFα, CCL3, CXCL12. En concreto IL8, CCL2, y CCL5 promueven la migración de las MSCs a través de los componentes que forman el factor de transcripción AP-1, un regulador indispensable de los procesos celulares de proliferación, diferenciación y apoptosis. Además de promover la migración de las MSCs, estos factores secretados por los macrófagos son capaces de alterar el perfil de expresión de citoquinas en las MSCs, concretamente aumentando la secreción de IL6, CCL5 y CXCL10[50].

- **Diferenciación:** Un estudio *in vivo* demostró que la infiltración de macrófagos precede a la actividad osteogénica en un ambiente ateroesclerótico, lo cual puso de manifiesto que la calcificación arterial es un proceso inflamatorio[51]. Así, por ejemplo, se ha demostrado que los macrófagos, tras ser activados, secretan oncostatina M (OSM), una citoquina de la familia de la IL6, que es capaz de inducir la mineralización de la matriz de las MSCs y su diferenciación a osteoblastos[52]. También se ha demostrado que el medio condicionado proveniente de macrófagos activados con IL1β es capaz de promover la diferenciación de MSCs a células de músculo liso[53].

Más recientemente, se ha visto que esta capacidad de diferenciación promovida por el medio condicionado de los macrófagos se debe en gran parte a que éste contiene exosomas que albergan mRNAs y miRNAs capaces de penetrar y ejercer su actividad biológica en otras células[54].

c) Secreción de factores tróficos.

Como ya dijimos, Son numerosos los estudios que sugieren que ciertas capacidades de las MSCs requieren su "activación" previa mediante su estimulación con factores proinflamatorios como IFNγ TNFα, IL1α, ó IL1β[55] procedentes del tejido dañado. La "activación" de las MSCs induce el aumento de la expresión de diversos tipos de moléculas como: **factores de crecimiento** (incluyendo factores proangiogénicos), **factores inmunosupresores** y por último **quimioquinas y moléculas de superficie.** Todas estas moléculas juegan un papel indispensable en la reparación del tejido dañado **(Figura 3).**

- **Factores de crecimiento:** Las MSCs producen varios **factores de crecimiento** como: EGF, FGF, PDGF, TGF*β*, VEGF, HGF, IGF1, ANGPT1, y CXCL12[56-59]. Estos factores promueven la generación de **células endoteliales, fibroblastos** (las células más abundantes de el tejido conectivo) **y otras células progenitoras** específicas del tejido.

En muchos casos, tras el daño en el tejido, la capa endotelial de los capilares se rompe, permitiendo la entrada de muchas proteínas de plasma y leucocitos de la sangre. Los factores de crecimiento liberados por las MSCs afectan directamente a las células endoteliales, promoviendo la angiogénesis a través de la proliferación de las mismas, produciendo matriz extracelular y reduciendo la permeabilidad endotelial. Además, los fibroblastos también colaboran en mantener la integridad del tejido mediante la secreción de proteínas de matriz extracelular y metaloproteínas de matriz epidermal como colágeno, gelatina o fibronectina.

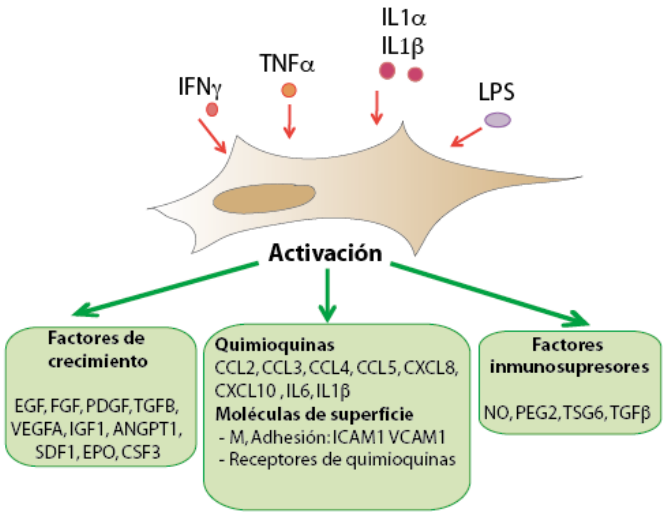

Figura 3. Secreción de factores paracrinos por MSCs. Los factores proinflamatorios secretados por el tejido dañado estimulan la secreción de diferentes tipos moléculas por parte de las hMSCs. Entre estas moléculas se encuentran: factores de crecimiento, quimioquinas, moléculas de superficie y factores inmunosupresores, las cuales en su conjunto contribuyen al proceso de reparación. Adaptado de Shi y cols.[56].

También se sabe que las MSCs producen otros factores, como eritropoyetina (EPO) y factor estimulante de colonias de granulocitos (CSF3), que median la supervivencia, proliferación y diferenciación de células madre hematopoyéticas, las cuales están implicadas en promover la reparación/regeneración de los tejidos no hematopoyéticos[60, 61].

- **Factores inmunosupresores:** Son moléculas capaces de inhibir o prevenir la activación del sistema inmune actuando de forma directa sobre las células de dicho sistema. Entre estos factores encontramos óxido nítrico, prostaglandina E2 e IL10. En un apartado posterior se discutirán más en detalle los mecanismos implicados en la modulación de la respuesta inmune por las MSCs.

- **Quimioquinas y moléculas de superficie:** Las MSCs expresan de forma constitutiva una gran variedad de quimioquinas y **receptores de quimioquinas**, que les permiten su movilización al lugar del daño. No obstante, se ha visto que la activación de las MSCs con TNFα ó IFNγ (solos o en combinación) contribuye a aumentar la expresión de **receptores de quimioquinas**[27].

c.1) Inmunorregulación.

La inmunorregulación es el proceso por el cual se controla la respuesta inmune mediada principalmente por las células T, B, macrófagos y células *natural killer* (NK). Las MSCs llevan a cabo esta inmunorregulación mediante diversos mecanismos que son activados por la presencia de factores proinflamatorios[55, 62] **(Figura 4**).

- **Oxido nítrico sintasa inducible (iNOS):** Las MSCs murinas expresan iNOS, la cual produce óxido nítrico (NO). En cambio, en MSCs humanas los niveles de esta enzima son muy bajos, y por el contrario expresan altas cantidades de IDO, como veremos a continuación. El NO producido es un potente inmunosupresor a través de una gran cantidad de mecanismos aún no completamente definidos. Así, por ejemplo, se sabe que en altas concentraciones, el NO es capaz de inhibir la proliferación de las células T *in vitro*[63].

- **Indoleamine 2,3-dioxygenase (IDO):** El papel de IDO, consiste en catalizar la degradación del triptófano (un aminoácido esencial para la proliferación de linfocitos), favoreciendo la producción de quinurenina. De esta forma IDO ha sido relacionado con la inhibición de la proliferación de varios tipos celulares, incluyendo células T, B y NK[64-66]**.** Esta enzima se expresa en humanos y en muy bajas cantidades en ratón.

- **Prostaglandina E2 (PGE2):** La prostaglandina E2 ha sido descrita como un potente supresor de células T, NK, y macrófagos[62, 64, 67]**.**

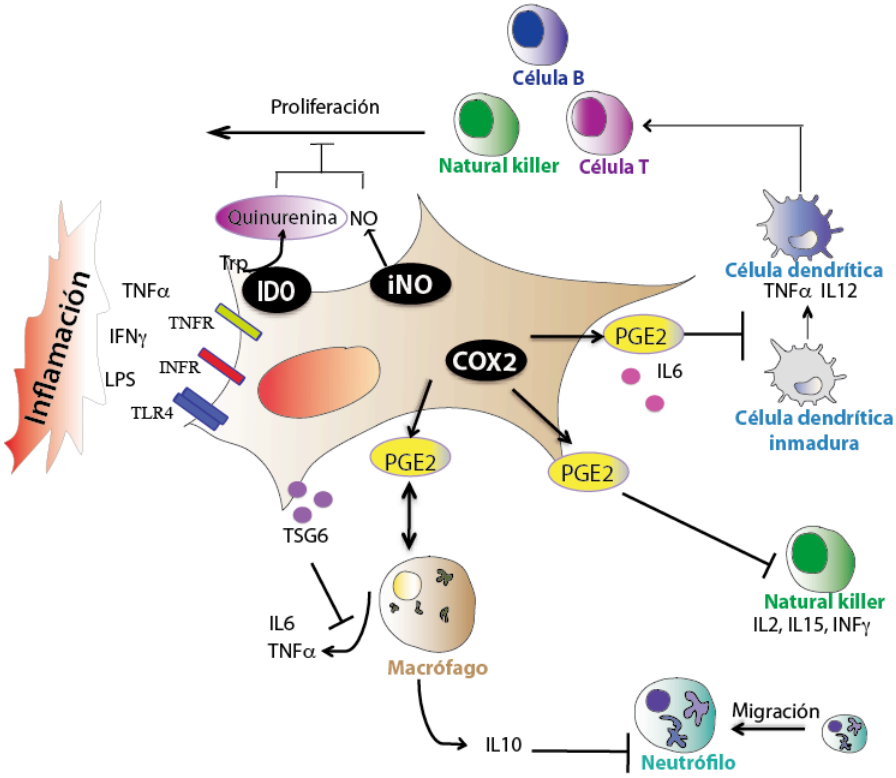

Figura 4. Modulación del sistema inmune mediada por las MSCs. Tras la inflamación, las MSCs son activadas por varias citoquinas proinflamatorias como TNFα e IFNγ, así como por otros factores asociados a procesos infecciosos, como LPS. En humanos, la activación de las MSCs produce un incremento en la expresión de IDO, lo cual aumenta la cantidad de quinurenina, que a su vez inhibe la proliferación de las células T y B. En ratón, sin embargo, esta activación promueve fundamentalmente la expresión de iNOS y la consiguiente producción de NO, la cual produce efectos antiproliferativos similares. A su vez, las MSCs activadas expresan COX2, que produce PGE2, la cual afecta a los macrófagos aumentando la secreción de IL10. Los altos niveles de PGE2 también bloquean la correcta maduración de las células dendríticas, lo que se traduce en una pobre activación de las células T y por tanto en una importante supresión de toda la respuesta inmune subsiguiente. Adaptado de Frenette y cols.[68].

PGE2 es un metabolito producido por la enzima prostaglandina-endoperóxido sintasa 2 (PTGS2), también llamada ciclooxigenasa 2 (COX2), que se induce mediante la activación de los receptores TLR4 de las hMSCs, los cuales reconocen moléculas de patógenos y de tejidos dañados. La activación de los TLRs induce también la secreción de citoquinas y quimioquinas, las cuales promueven el reclutamiento de las células del sistema inmune aumentando la función inmunorreguladora de las MSCs[63]. La PGE2 producida es capaz de educar a los macrófagos *in vivo*, cambiando su fenotipo de un estado proinflamatorio (elevada secreción de IL6 y baja de IL10) a un estado antiinflamatorio (alta secreción de IL10 y baja IL6) contribuyendo a la atenuación de la sepsis en ratón[62]. Esta elevada secreción de IL10 de los macrófagos previene la migración de neutrófilos a los lugares de daño.

En humanos se ha visto que PGE2 actúa en combinación con IDO, alterando la proliferación de las células T, así como la proliferación, citotoxicidad y producción de citoquinas (como IL2, IL15 e IFNγ) por las células NK. Además, también ha sido descrito que PGE2, junto con la IL6 secretada también por las MSCs, es capaz de inhibir la maduración de las células dendríticas[69, 70], las cuales tienen un papel fundamental en la presentación de antígenos a las células T.

- **IL10:** Ha sido implicada en el efecto inmunosupresor de las MSCs. Se sabe que el contacto con MSCs induce la producción de IL10 en las células presentadoras de antígeno como células dendríticas y monocitos-macrófagos[67, 71]**.**

- **TSG6/TNFAIP6:** TGS6 (*Tumor necrosis factor-inducible gene-6*) es una proteína supuestamente antiinflamatoria que se ha encontrado altamente expresada en una gran variedad de pacientes con enfermedades autoinmunes o inflamatorias. Todavía se requieren más investigaciones para conocer su mecanismo de acción concreto, aunque un estudio de este mismo año sugiere que es secretada por las MSCs y es capaz de disminuir los niveles de TNFα producidos por los macrófagos, reduciendo la inflamación y la fibrosis[72].

- **Quimioquinas y sus receptores:** Los factores inmunosupresores secretados por las MSCs son muy lábiles y únicamente tienen efecto en proximidad de las MSCs que las secretan. Por esta razón, las MSCs también producen **quimioquinas,** como los ligandos de CXCR3 (receptores presentes en las células T y *natural killers*), y de CCR5 (expresado en células Th1), que sirven a las MSCs para atraer a las células del sistema inmune. Además, el tratamiento de las MSCs con IFNγ es capaz, a través de sus receptores TLR, de aumentar la expresión de citoquinas proinflamatorias como IL1β, IL6, y de quimioquinas como IL8 ó CCL5, todas las cuales son capaces de atraer a los neutrófilos al lugar del daño[73].

Una vez que las células del sistema inmune han sido atraídas, es necesario retenerlas, por ello las MSCs también expresan **moléculas de superficie,** entre las que se encuentran moléculas de adhesión (ICAM1 y VCAM1) y receptores de quimioquinas. Las MSCs expresan ICAM1 y VCAM1 para reclutar a las células T[74]. De esta manera se crea un microambiente que permite la amplificación de la acción de los distintos factores inmunorreguladores producidos por las MSCs.

- **Otros mediadores:** Otros moduladores del sistema inmune secretados por las MSCs (y también por otras células madre adultas o progenitoras) después de la estimulación inflamatoria, son: PDL1 (del ingés *programmed death ligand* 1), HO1 (del inglés *heme oxygenase*-1), LIF (del inglés *leukemia inhibitory factor*). Todos ellos son capaces de reducir la proliferación de las células T, aunque sus mecanismos de acción son aún muy poco comprendidos.

Interacciones célula-célula.

Además de todos estos factores secretados por las MSCs, diversas evidencias indican que este proceso de inmunorregulación también es mediado por contacto directo entre MSCs y células del sistema inmune. Así, por ejemplo, se sabe que las MSCs en contacto directo con los macrófagos son capaces de estimular la producción de IL-10, lo que no ocurre cuando se utiliza solo el medio secretado por las MSCs[62]. También ha sido demostrado que el contacto directo de MSCs-macrófagos es capaz de aumentar la cantidad del marcador de superficie CD206, propio de macrófagos antiinflamatorios[75]. Por último, se ha descrito que la capacidad de inhibir la proliferación de las células T puede deberse en parte al contacto de proteínas de superficie de las MSCs, como HLA-G1[76], PDL1[77], CD200[78,79] y B7-H4[80], con sus respectivos receptores en las células T.

Aplicaciones clínicas de las MSCs.

Las propiedades terapéuticas únicas de las MSCs, junto con el hecho de que son fácilmente aislables y pueden ser expandidas *ex vivo*, hacen de estas células unas candidatas ideales para su uso en numerosos tratamientos clínicos.

El primer ensayo clínico en el que se usaron MSCs expandidas *ex vivo* data del año 1995[81]. Desde entonces, el número de ensayos clínicos ha ido aumentado continuamente. Actualmente, en la base de datos de los *U.S. National Institutes of Health* (http://clinicaltrials.gov) aparecen 379 ensayos usando MSCs en todo el mundo, para el tratamiento de diversas enfermedades; la **Tabla 1** ilustra los principales ejemplos.

Para el uso de éstas células en clínica resulta imprescindible su expansión en cultivo, ya que el número de MSCs necesario para estos tratamientos es muy superior al de células que pueden obtenerse directamente de una biopsia de tejido. Para hacernos una idea, actualmente en el tratamiento de la esclerosis múltiple se usan del orden de uno a dos millones de MSCs por cada kg que pese el paciente. Desafortunadamente, como explicaremos en el siguiente apartado, las MSCs cultivadas durante periodos prolongados de tiempo pueden ver significativamente disminuida o incluso suprimida su actividad terapéutica. Además, la edad de los donantes a partir de los cuales son aisladas las MSCs también se relaciona inversamente con su eficacia terapéutica[82].

Patología	Fuente de MSCs	Referencia	Patología	Fuente de MSCs	Referencia
Osteogénesis imperfecta	MO	83	**Enfermedad aguda y crónica de injerto contra huésped**	MO	84
	MO	85		MO	86
	F	87	**Infarto de miocardio**	MO	88
Defectos en el cartílago	MO	89		MO	90
	MO	91		MO	92
	MO	93		MO	94
	MO	95		MO	96
Trasplante de células madre hematopoyéticas	MO	97		MO	98
	MO	99		MO	100
	MO	101		Placenta	102
	MO	103	**Enfermedad de Crohn**	TA	104
	MO	105		TA	106
Enfermedad aguda de injerto contra huésped	MO	107	**Esclerosis múltiple**	MO	108
	TA	109		MO	110
	MO	111		MO	112
	MO	113		TA	114
	MO	115	**Lupus sistémico eritematoso**	MO	116
	MO	117		CU	118
	CU	119		CU	120
	MO	121		MO	122
	MO	123	**Cirrosis**	MO	124
Enfermedad crónica de injerto contra huésped	MO	125		MO	126
	MO	127			

Tabla 1. Principales ensayos clínicos usando MSCs. Se muestra el tipo de patología tratada con las MSCs, la fuente de donde se obtienen las MSCs, y las correspondientes referencias. MO (médula ósea), F (tejidos fetales), CU (cordón umbilical), TA (tejido adiposo).

2. SENESCENCIA.

Envejecimiento y senescencia.

De acuerdo a la definición propuesta por Sames y Stolzing en 2005, el envejecimiento es "la suma de todas las restricciones en los mecanismos de regeneración de los organismos multicelulares"[128]. Los tejidos de un organismo están regenerándose de manera continua (homeostasis)[129, 130], gracias a las células madre, lo cual subraya la implicación de éstas en el proceso de envejecimiento.

Debido al hecho de que las células madre están implicadas en el proceso de homeostasis, así como en la reparación y regeneración de numerosos tejidos, el envejecimiento se podría entender como una pérdida de la funcionalidad de las células

madre que residen en dichos tejidos. Por ello, la disminución de la funcionalidad de las células madre se relaciona con la edad. Algunos trabajos han aislado células madre de individuos jóvenes y viejos, y las han transferido a ambientes idénticos tanto *in vitro* como *in vivo*, observando que las células provenientes de donantes de más edad son menos funcionales.

En los años sesenta se propuso el concepto de senescencia replicativa, el cual consiste en que las células somáticas pueden llevar a cabo un número limitado de divisiones antes de entrar en un estado (senescencia replicativa) en el que la célula deja de dividirse. Este fenómeno fue descrito por Leonard Hayflick, por lo que a dicho número limitado de divisiones también se le denominó "limite de Hayflick"[131]. Aunque la senescencia replicativa fue primeramente caracterizada *in vitro*, también ha sido propuesta como un mecanismo que ocurre *in vivo*.

Hoy en día no está del todo claro si las células madre provenientes de individuos de avanzada edad presentan un fenotipo más próximo a la senescencia por mecanismos intrínsecos a la propia célula, o por señales del propio microambiente que las rodea. Sin embargo, en cuanto a la senescencia celular *in vitro*, se sabe que es inducida por varios estímulos, los cuales incluyen la erosión de los telómeros, la activación de oncogenes, el estrés oxidativo, condiciones de cultivo subóptimas y agentes que dañan el DNA o la estructura de la cromatina[132-135]. Por tanto, el mantenimiento de las células en cultivo aumenta en la práctica su envejecimiento.

Marcadores de senescencia de las MSCs.

Las células mesenquimales, al igual que todas las demás células somáticas, alcanzan la senescencia tras un determinado número de divisiones en cultivo. Dicha senescencia se caracteriza por una serie de alteraciones fenotípicas (**Figura 5**), entre las cuales se encuentran:

(1) **Aumento de tamaño**: las MSCs senescentes son mucho más grandes que las jóvenes[136-138]; además, dejan de presentar una morfología con forma de aguja (propia de células jóvenes) y adquieren una morfología más redondeada[139]. **(2) Presencia de actividad beta-galactosidasa:** la actividad de esta enzima aumenta tras periodos largos de cultivo[138, 140]. Sin embargo, no hay un consenso claro sobre si este marcador incrementa con la edad de los donantes de donde se obtienen estas células[138, 141]. **(3) Arresto proliferativo**: se caracterizada por el arresto del crecimiento en la fase G1, produciendo la represión de genes que conducen la progresión del ciclo celular y el aumento de la

expresión de inhibidores del ciclo, como p53/p21 y p16/RB. Hoy en día parece haber un consenso sobre que la senescencia ocurre de forma excluyente vía una u otra ruta: p53 media fundamentalmente la senescencia debida a la disfunción de los telómeros y daño en el DNA, mientras que la ruta p16 mediaría principalmente la senescencia debida a oncogenes, daños en la cromatina y diferentes tipos de estrés[142]. **(4) Fenotipo secretor asociado a senescencia**: Varios estudios confirman que las células senescentes activan un fenotipo secretor asociado a senescencia conocido como SASP (del inglés, *Senescence-Associated Secretory Phenotype*). El SASP se caracteriza por el aumento de la secreción de numerosas citoquinas proinflamatorias, que incluyen: IL8, IL6, GMCSF, GROα, GROβ, GROγ, ICAM1 y MCP1/4. *In vivo*, ha sido confirmado el aumento en la expresión de IL6 en hMSCs con la edad[143]. IL6 es una citoquina capaz de regular la proliferación, diferenciación y la actividad de varios tipos celulares[144], además varios estudios han confirmado que el aumento de los niveles en suero de IL6 pueden ser indicadores de mortalidad en pacientes de edad avanzada[145-147]. (6) **Pérdida de funcionalidad**: Las MSCs senescentes muestran una actividad biológica reducida, como veremos más en detalle en el siguiente apartado.

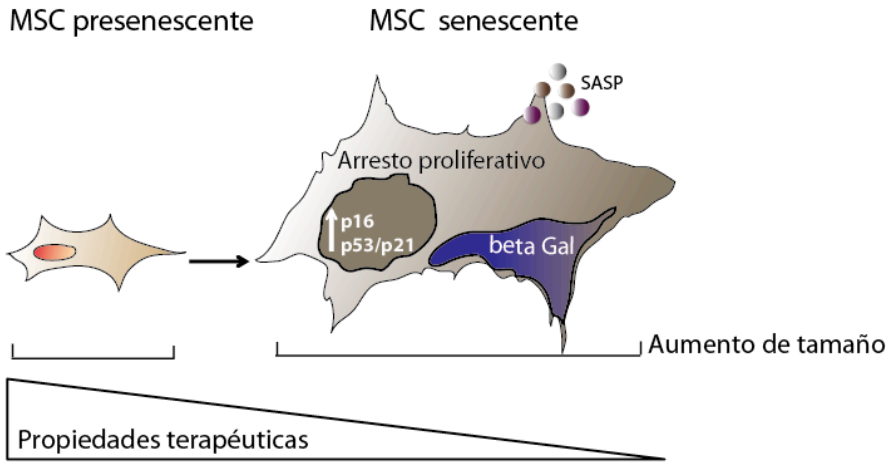

Figura 5. Diferencias entre MSCs presenescentes y senescentes. Las MSCs senescentes se caracterizan por presentar un tamaño mayor, arresto proliferativo mediado por las rutas de p53/p21 ó p16, un incremento de su fenotipo secretor y por la expresión de beta-galactosidasa a pH 6. Además, a medida que las MSCs van envejeciendo se produce una disminución en sus propiedades terapéuticas. Adaptado de Rodier y Campisi[148].

Papel de la senescencia en las propiedades terapéuticas de las MSCs.

Numerosos estudios han demostrado que la senescencia de las MSCs conlleva la limitación de sus propiedades terapéuticas. Las conclusiones de dichos estudios pueden resumirse en las siguientes:

- El cultivo prolongado *ex vivo* de las MSCs hace que la cantidad de receptores de quimioquinas y moléculas de adhesión presentes en su superficie disminuyan, incluso hasta llegar a desaparecer[28]. Asimismo, se ha visto mediante estudios *in vivo* que el potencial de migración de las MSCs trasplantadas, hacia los sitios de daño tisular disminuye a medida que estas células han permanecido más tiempo en cultivo[149].

- La capacidad de proliferación de las MSCs correlaciona negativamente con la edad de los donantes de donde provienen[136, 150] .

- Se ha demostrado que las MSCs van perdiendo de manera gradual su potencial de diferenciación en condiciones de cultivo prolongado[151, 152], lo cual se asocia, entre otros factores, a metilaciones en el DNA de genes implicados en el proceso de diferenciación[153, 154].

- La producción de las moléculas que median la inmunorregulación disminuye en las MSCs senescentes[155, 156].

Todos estos datos revelan que la senescencia es potencialmente capaz de suprimir las capacidades terapéuticas de las MSCs; de ahí que sea de gran importancia determinar el grado de envejecimiento de estas células para controlar la calidad de las preparaciones celulares de uso terapéutico[157, 158]. Por este motivo, el estudio de los mecanismos que regulan el proceso de senescencia en las células madre mesenquimales es crítico para mejorar su uso en la medicina regenerativa.

3. miRNAs.

Definición.

Los miRNAs son pequeñas moléculas de RNA no codificante (entre 21-25 nt) que actúan regulando la expresión génica. Fue en el año 1993 cuando se describió por primera vez en *Caenorhabditis elegans* la existencia de un gen denominado *lin-4* que codificaba un pequeño RNA con complementariedad hacia el gen *lin-14*, capaz de disminuir la expresión de la proteína LIN-14[159]. Durante años se creyó que se trataba de un mecanismo único,

hasta el descubrimiento de let-7, el cual reprime la expresión de *lin-41*, *lin-14*, *lin-28*, *lin-42*, y *daf-12* durante el desarrollo de *C. elegans*[160].

La identificación de homólogos de let-7 en varias especies de vertebrados incluyendo humanos despertó un gran interés por estas moléculas, demostrándose que estos pequeños RNAs (posteriormente denominados microRNAs ó miRNAs) están conservados en la evolución y son expresados de forma ubicua en todos los organismos eucariotas[161-163].

Biogénesis y mecanismos de regulación.

Los miRNAs se originan a partir de moléculas precursoras de tamaño variable de doble cadena, con bucles a modo de horquilla, capaces de originar RNA con estructuras secundarias. Aproximadamente el 80% de los genes de miRNAs se encuentra localizado en regiones intrónicas del genoma y el 20% restante se distribuye en regiones exónicas y regiones intergénicas.

Alrededor de la mitad de los genes de miRNA se encuentra formando parte de familias (miRNAs que comparten la secuencia que se une a la región complementaria del RNA mensajero diana, conocida como región semilla) o grupos génicos (*clusters* de miRNAs) formados por miRNAs localizados en la misma región dentro del genoma[164]. Al compartir la región semilla, los miembros de una misma familia de miRNAs son potenciales reguladores del mismo conjunto de genes. En humanos se han identificado hasta el momento 1.872 secuencias distintas de miRNAs (base de datos miRBase) y más de 18.000 interacciones entre miRNA-mRNA[165].

Mecanismos de regulación: Después de ser transcritos, los miRNAs primarios son procesados en el núcleo por complejos de proteínas que contienen la endonucleasa Drosha, dando lugar a precursores (pre-miRNA) que seguidamente son exportados al citoplasma.

Una vez en el citoplasma, la endonucleasa Dicer corta el pre-miRNA, generando un dúplex, que es incorporado al complejo de silenciamiento inducido de RNA (RISC). Una hebra es retenida por este complejo, la cual constituye el miRNA maduro que se une al(los) mRNA(s) diana(s), causando la inhibición de su traducción. Cada miRNA reconoce e inhibe potencialmente a cientos de mRNA parcialmente complementarios, modulando así de forma muy potente la expresión génica de la célula.

Además de su función intracelular, en los últimos años diversos trabajos han demostrado que los miRNAs son transportados en grandes cantidades en exosomas, lo que convierte a estas moléculas también en mecanismos de comunicación intercelular[166].

Durante la última década los miRNAs han sido extensamente investigados, y se ha demostrado que controlan procesos biológicos críticos, como el desarrollo y la homeostasis de tejidos, además de encontrarse directamente implicados en numerosas patologías.

Relación entre miRNAs y células madre mesenquimales.

El estudio de los miRNAs añade una nueva dimensión al estudio de la regulación de las funciones de las células madre. Ambos campos han convergido con la identificación de miRNAs específicos de este tipo de células[167]. A partir de las observaciones iniciales que indicaban que los miRNAs parecían regular en gran medida el destino de las células madre y su comportamiento, controlando simultáneamente un gran número de genes, se ha demostrado que los miRNAs son capaces de activar o inhibir enteramente procesos complejos tales como la diferenciación o la proliferación **(Tabla 2).**

miRNAs y senescencia.

La importancia general de los miRNA en la senescencia celular quedó establecida cuando se encontró que el *knock-out* de Dicer inducía senescencia[168]. Durante los últimos años, diversos estudios han identificado varios miRNAs implicados en el proceso de senescencia en distintos tipos celulares, incluyendo fibroblastos[169, 170] y MSCs de distintos orígenes[171, 172].

Papel de los miRNAs en la senescencia de las MSCs.

Muchos miRNAs todavía no caracterizados podrían ser regulados durante el proceso de senescencia y/o contribuir a un fenotipo asociado a la misma. En los últimos años (de forma simultánea a la realización del trabajo descrito en la presente Tesis Doctoral) varios estudios han relacionado la expresión de varios miRNAs con la senescencia en MSCs.

Así, por ejemplo, en MSCs de tejido adiposo, la expresión de miR-486-5p aumenta de forma progresiva en cultivo, y su sobreexpresión produce la adquisición de un fenotipo prematuro de senescencia que incluye la inhibición de la proliferación y la diferenciación a tejido adiposo y óseo. Se cree que este miRNA regula la expresión de SIRT1, el principal regulador conocido de la longevidad y desórdenes metabólicos asociados a la edad[172]. Otro miRNA relacionado con senescencia en MSCs es miR-10, cuya expresión es menor en las células aisladas de pacientes de edad avanzada en comparación con pacientes jóvenes. La regulación a la baja de este miRNA reduce la diferenciación y aumenta la senescencia a

través de la regulación del factor de transcripción KLF4 (del inglés *Kruppel-like factor 4)* [173].

Por otro lado, ha sido descrito que ZMPSTE24 es responsable de la acumulación de la laminina A en hMSCs senescentes, siendo diana directa del miR-141-3p, el cual se sobreexpresa durante la senescencia replicativa mediante regulación epigenética[174].

Función	miRNA	MSCs	Papel	Referencia
Condrogénesis	miR-574-3p	hMO	Modulador de la condrogénesis	175
	miR-23b	hMO	Promueve condrogénesis	176
Adipogénesis	miR-124	hMO	Favorece la diferenciación a tejido adiposo	177
	miR-369-5p	hMO	Inhibe la adipogénesis	178
	miR-371	hMO	Promueve la adipogénesis	178
Osteogénesis	miR-29b	hMO	Aumenta la osteogénesis	179
	miR-21	mMO	Disminuye en osteoporosis	180
	miR-17	hMO	Aumenta la osteogénesis	181
	miR-31	hMO	Regulador negativo de la osteogénesis	182
Actividades inmunorreguladoras	miR-27b	hTA	Inhibe propiedades tolerogénicas	183
	miR-27b	mMO	Inhibe la migración	184
	miR-146a	hMO	Aumenta la respuesta inflamatoria	185
Proliferación , diferenciación y apoptosis	miR-32-5p	rMO	Regula PI3K/Akt	186
	miR-148a	hCU	Suprime apoptosis	187
	miR-21	hMO	Protege de la apoptosis producida por hipoxia	188
	miR-23a	hMO	Protege de la apoptosis producida por hipoxia	188
	miR-96	hMO	Favorece la diferenciación	177
	miR-199a	hMO	Favorece la diferenciación	177
	miR-193	hMO	Favorece la proliferación	189
	miR-143	rMO	Progresión de ciclo celular	190
	miR-125b	hMO	Protege de anoikis	191
	miR-503	hMO	Protege de la apoptosis producida por hipoxia	188
Estado inmaduro y pluripotente	miR-335	hMO	Su down-regulación es necesaria para la diferenciación	192
Transdiferenciación	miR-133b	rMO	Regula el crecimiento de neuritas	193
	miR-9	mMO	Promueve neurogénesis	194
	miR-34a	hCU	Aumenta la neurogénesis	195
Otras	miR-21^{GOF}	hTA	Regula la actividad antitumoral	196
	miR-126b^{GOF}	hMO	Aumenta angiogénesis en isquemia	197
	miR-145^{GOF}	rMO	Respuesta a TGF-β1 e hipoxia	198

Tabla 2. miRNAs implicados en la biología de las MSCs. En esta tabla se muestran los principales miRNA encargados de la regulación de diferentes procesos en MSCs obtenidas de distintas fuentes**.** m, ratón; r, rata; h, humano; MO, médula ósea; TA, tejido adiposo; CU, cordón umbilical; GOF (del inglés *gain of function).*

Otros estudios comparan el perfil de expresión de los miRNAs y mRNAs entre MSCs aisladas de donantes de avanzada edad y aisladas de donantes jóvenes. Los resultados muestran numerosos cambios en el perfil de expresión de miRNA entre los que cabe destacar el aumento de miR-335, el cual será objeto de nuestro estudio. En cuanto al perfil de mRNA, se observa una disminución en las MAPKs (del inglés *mitogen-activated protein kinase*) p38 y ERK1/2, así como los componentes del complejo AP-1, c-FOS y c-JUN[199].

miR-335 *

La secuencia que codifica miR-335 se localiza en el intrón 2 del gen murino Mest (y su homólogo humano MEST) y codifica para una enzima altamente conservada perteneciente a la familia de las alfa/beta hidrolasas. Mest, también conocido como Peg1, fue primeramente identificado como un gen con *imprinting* paterno[200]. Su elevada conservación a lo largo de la evolución contrasta con el *imprinting* del locus, el cual sólo se conserva en marsupiales y mamíferos placentarios. Hasta la fecha miR-335 es de los pocos genes de miRNA conocidos con *imprinting*.

Los estudios realizados han demostrado que el patrón de expresión de Mest incluye principalmente el mesodermo embrionario y el cerebro en desarrollo; sin embargo dicha expresión es muy baja o casi nula en tejidos adultos[200-202]. Durante el último año se ha descrito que la expresión de miR-335 promueve la diferenciación a mesodermo e inhibe la formación del ectodermo gracias a la represión de factores de transcripción como SOX17 y Fox2[203].

Inicialmente, Tavazoie observó que este miRNA era capaz de inhibir la invasión de células metastásicas, y lo identificó como un supresor de metástasis en cáncer de mama[204]. Un estudio posterior encontró una deleción genética de miR-335 y una hipermetilación del promotor de Mest, ambas capaces de silenciar miR-335, que se producen en pacientes con cáncer con elevado potencial metastático[205]. Otros trabajos muestran que la desregulación de miR-335 se asocia a varios tipos de cáncer en humanos como carcinoma hepatocelular[206-208], mieloma múltiple[206], cáncer de ovario[208] y carcinoma celular renal[209].

También se ha observado que miR-335 es uno de los miRNAs más regulado a la baja en células tumorales cocultivadas con macrófagos TAM, una subpoblación de macrófagos asociada a tumores que, una vez activados crean un microambiente que estimula el

* La nomenclatura oficial de este miRNA es: humano, hsa-miR-335-5p; ratón, mmu-miR-335-5p; rata, rno-miR-335-5p. Para simplificar, y dado que todos ellos tienen la misma secuencia, en esta tesis denominaremos a todos simplemente como "miR-335".

crecimiento del tumor, su invasión y metástasis. Lo cual podría constituir un mecanismo que contribuya a la invasividad de las células tumorales[210].

Más recientemente, como comentaremos extensamente en el apartado de Discusión, diversos trabajos han relacionado a miR-335 con procesos de envejecimiento y senescencia celular.

En el presente trabajo, identificamos a miR-335 como un regulador clave de la activación de las hMSCs, y demostramos su participación en el proceso de senescencia, con importantes implicaciones en la funcionalidad biológica y la eficacia terapéutica de estas células.

OBJETIVOS

Desde hace relativamente poco tiempo, los miRNAs han emergido como potentes reguladores capaces de modular la expresión de cientos de genes de manera simultánea. Se sabe que los miRNAs juegan un papel esencial en la biología de las células madre, regulando procesos clave como la diferenciación o la autorrenovación. Sin embargo, el conocimiento sobre los miRNAs encargados de regular el potencial terapéutico de las hMSCs es escaso. Este conocimiento podría convertirse en una importante herramienta para controlar y mejorar la funcionalidad de las hMSCs usadas en clínica.

Los objetivos de esta tesis fueron:

1. **Identificar los principales miRNAs implicados en la regulación de la transición entre el estado inactivo o de reposo y el estado reparativo de las hMSCs.**

2. **Estudiar las principales señales que median la regulación de la expresión de dichos miRNAs en las hMSCs.**

3. **Establecer el efecto que tienen dichos miRNAs sobre las diferentes actividades terapéuticas de las hMSCs.**

4. **Estudiar las rutas/mecanismos mediante los cuales, dichos miRNAs regulan la actividad terapéutica de las hMSCs.**

MATERIALES, MÉTODOS Y RESULTADOS

1. miR-335 controla la proliferación, migración y diferenciación de las células madre mesenquimales humanas (Tomé *et al.,* Cell Death Differ. 2011; 18:985-996).

El objetivo de este artículo se centró en la identificación de los miRNAs implicados en el control de la activación de las hMSCs. Para ello, partimos de la hipótesis de que dichos miRNAs deberían estar presentes en el estado inactivo o indiferenciado de las hMSCs, y su expresión debería disminuir tras la activación de las mismas, permitiendo la iniciación de distintos programas biológicos, como la diferenciación.

Primeramente comparamos hMSCs en su estado indiferenciado o inactivo (hMSCs cultivadas en medio de expansión) con hMSCs activadas (hMSCs cultivadas en medio que induce diferenciación a grasa o a hueso) y fibroblastos dérmicos, los cuales son comúnmente considerados como un tipo celular mesenquimal totalmente diferenciado. Mediante estudios de expresión génica con microarrays, encontramos que miR-335 fue el único miRNA regulado a la baja de forma significativa durante el proceso de diferenciación en las tres condiciones ensayadas, en comparación con las condiciones control. Además, tanto miR-335 como su gen hospedador, MEST, se mantuvieron expresados a mayores niveles en hMSCs obtenidas de diferentes fuentes, en comparación con los fibroblastos.

También encontramos que la expresión de miR-335 es regulada por señales que controlan la biología de las hMSCs. Así, la expresión de miR-335 disminuyó en presencia de señales que inducen la activación de las hMSCs, como IFNγ, una citoquina proinflamatoria que en las hMSCs induce fenotipo inmunorregulador. Por el contrario, el tratamiento de las hMSCs con Wnt3a, el cual promueve el mantenimiento de las hMSCs en sus nichos e inhibe diferenciación, provocó el aumento de la expresión de miR-335.

Además, la expresión exógena forzada de miR-335 en las hMSCs produjo la inhibición de su capacidad de proliferación, migración y diferenciación a tejido adiposo y hueso *in vitro*. Por último, fuimos capaces de identificar RUNX2, un factor de transcripción crítico en la diferenciación osteogénica, como una diana directa de miR-335.

En conjunto, todos estos resultados nos permitieron proponer el papel central de miR-335 en la regulación de la biología de las hMSCs, manteniendo un estado de inactivación y probablemente restringiendo/limitando su potencial terapéutico.

Cell Death and Differentiation (2011) 18, 985–995

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miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells

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In spite of the extensive potential of human mesenchymal stem cells (hMSCs) in cell therapy, little is known about the molecular mechanisms that regulate their therapeutic properties. We aimed to identify microRNAs (miRNAs) involved in controlling the transition between the resting and reparative phenotypes of hMSCs, hypothesizing that these miRNAs must be present in the undifferentiated cells and downregulated to allow initiation of distinct activation/differentiation programs. Differential miRNA expression analyses revealed that miR-335 is significantly downregulated upon hMSC differentiation. In addition, hMSCs derived from a variety of tissues express miR-335 at a higher level than human skin fibroblasts, and overexpression of miR-335 in hMSCs inhibited their proliferation and migration, as well as their osteogenic and adipogenic potential. Expression of miR-335 in hMSCs was upregulated by the canonical Wnt signaling pathway, a positive regulator of MSC self-renewal, and downregulated by interferon-γ (IFN-γ), a pro-inflammatory cytokine that has an important role in activating the immunomodulatory properties of hMSCs. Differential gene expression analyses, in combination with computational searches, defined a cluster of 62 putative target genes for miR-335 in hMSCs. Western blot and 3′UTR reporter assays confirmed *RUNX2* as a direct target of miR-335 in hMSCs. These results strongly suggest that miR-335 downregulation is critical for the acquisition of reparative MSC phenotypes.
Cell Death and Differentiation (2011) **18,** 985–995; doi:10.1038/cdd.2010.167; published online 17 December 2010

Mesenchymal stem cells (MSCs) are multipotent mesoderm-derived somatic stem cell (SSC) precursors of non-hematopoietic connective tissues that are present in the stroma of virtually all mammalian organs, especially bone marrow and subcutaneous fat (reviewed in Bernardo *et al.*[1]). Upon activation by tissue damage, MSCs contribute to tissue-repair processes through a multitude of activities, including cell proliferation, differentiation and migration, and the regulation of angiogenesis and immune responses. There is growing evidence, in both animal and clinical models, that administration of *ex vivo*-expanded human MSCs (hMSCs) has potential to ameliorate many degenerative disorders; however, the specific molecular mechanisms underlying this therapeutic potential remain mostly unknown.

MicroRNAs (miRNAs) are an extensive family of small (18–24 nucleotide), single-stranded non-coding RNAs, which regulate gene expression in eukaryotic cells by controlling the translation (usually by repression), stability and localization of specific mRNA targets. Computational predictions of miRNA targets indicate that each miRNA regulates hundreds of mRNAs, and that approximately one third of all mammalian protein-coding genes are regulated by miRNAs.[2] Functional studies show that miRNAs participate in virtually each cellular process investigated, and that alterations in their expression levels might underlie human diseases, including cardiovascular disease and cancer. There are also data indicating that mammalian miRNAs can be imported into the nucleus[3] or even secreted from cells within small exocytic particles,[4] suggesting the existence of currently unknown functions for this class of molecules.

Experimental evidence shows that miRNAs are critical regulators of stem cell biology. In mouse embryonic stem (ES) cells, knockout of Dicer or DGCR8, two key protein factors involved in miRNA biogenesis, impairs cell growth, with cells accumulating in G1, and severely reduces differentiation capacity.[5,6] Additionally, expression profiling demonstrates that ES cells express a unique repertoire of miRNAs compared with differentiated cell types.[7] Molecular functions of ES cell-specific miRNAs have begun to be elucidated, and the results confirm their critical involvement in the regulation of ES cell pluripotency and self-renewal. miRNAs also have an important role in controlling ES cell differentiation and commitment.[8]

To date, relatively few studies have examined miRNA function in MSCs. Gene expression microarray profiling has identified several miRNAs that are regulated during differentiation of MSCs into different cell lineages. Some of these miRNAs have been identified as regulators of osteogenic differentiation, including miR-125b,[9] miR-26a,[10] miR-196a,[11] miR-204/211[12] and miRs-148b, -27a and -489.[13] Also, miR-335 has been recently identified as the most upregulated miRNA in bone marrow-derived hMSCs in comparison with

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Keywords: microRNA; mesenchymal stem cell; cell proliferation; cell migration; cell differentiation; osteogenesis
Abbreviations: hMSC, human mesenchymal stem cells; fdr, false discovery rate
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skin fibroblasts,[14] which are otherwise phenotypically similar to hMSCs. However, the specific targets of most of these miRNAs remain to be determined, and the possible role of miRNAs in other therapeutically relevant MSC activities, such as in cell migration and proliferation, is unknown.

Here, we sought to identify miRNAs that may regulate the activation of the reparative phenotype of hMSCs, as well as some of their target genes.

Results

miR-335 is downregulated upon hMSC differentiation. We hypothesized that key miRNA regulators of MSC activation/differentiation in tissue repair should be expressed in the undifferentiated state, and downregulated early upon exposure to a differentiation signal. To identify these putative miRNAs, we used Agilent microarrays to obtain differential miRNA expression profiles of undifferentiated bone marrow-derived hMSCs *versus* the same cells cultured in the presence of adipogenic or osteogenic media. We also profiled human skin fibroblasts, since the target miRNAs should be expressed at comparatively low levels in more developmentally restricted mesenchymal cell types. As we aimed to identify miRNAs potentially involved in the initial steps of hMSC activation/differentiation, cells were exposed to differentiation media for a relatively short period (9 days), instead of the 21 days commonly used for *in vitro* MSC differentiation assays.

Signal processing is a critical step in the analysis of the results of miRNA microarray experiments. We used a normalization algorithm that incorporates quantile normalization between arrays[15] to estimate a processed miRNA signal for the Agilent arrays. The quantile normalization, when applied to the background-corrected signal, showed significantly lower variability between replicates than the total gene signal normalized by the 75% percentile (Supplementary Figure S1).

The results showed no significant regulation (false discovery rate, fdr$<$15%) of miRNAs previously described as regulators of osteogenic (miR-26a, miR-27a, miR-125b, miR-148b, miR-196a and miR-489) or adipogenic differentiation (miR-103, miR-107 and miR-143) under any of the conditions tested (Supplementary Figure S2A; Supplementary Table S1). Gene enrichment analysis of the predicted targets of miRNAs up- or downregulated in at least two conditions (see Materials and methods) showed a significant ($P$$<$1E–06) enrichment for genes involved in angiogenesis or signaling by Wnt, integrins, PDGF, cadherin, endothelin and TGF-β (Supplementary Figure S2B; Supplementary Table S1). miR-335 was the only miRNA significantly downregulated in all three 'differentiated' cell populations (Figure 1a). Fold-change (log2) values were as follows: fibroblast *versus*

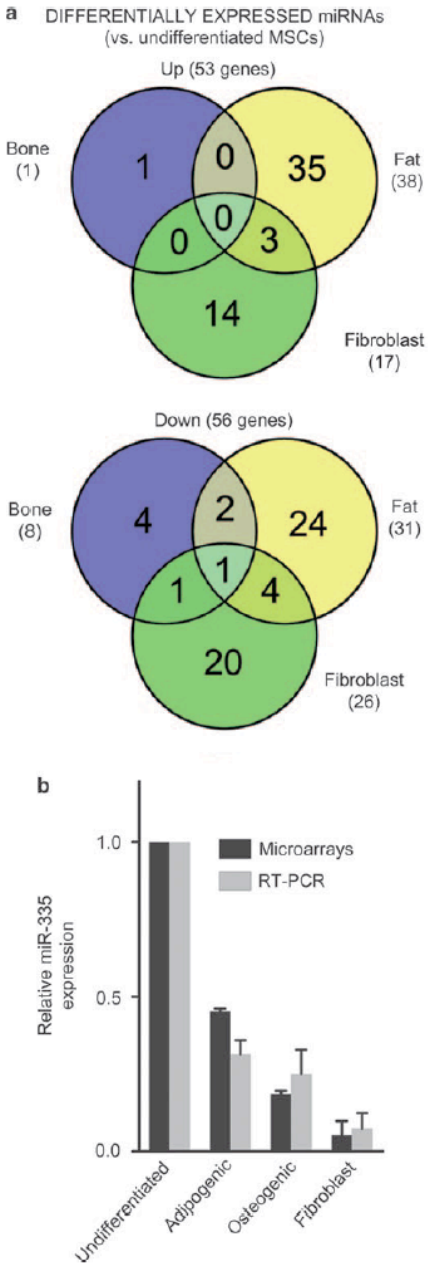

Figure 1 miR-335 is downregulated in differentiated hMSCs and primary skin fibroblasts. (**a**) Total RNA was isolated from four bone marrow-derived MSC samples grown for 9 days in expansion medium or medium containing osteogenic or adipogenic supplements. In parallel, total RNA was also isolated from four dermal fibroblasts samples. The Venn diagrams show the number of miRNAs differentially expressed (fdr$<$15%) in each differentiated cell population, analyzed on Agilent V2 miRNA arrays. For fibroblasts, samples (N=4) were analyzed individually, whereas for osteogenic and adipogenic differentiation, samples (N=4) were pooled (two pools, two independent samples each) before microarray analysis. (**b**) Real-time RT-PCR validation of the microarray results for miR-335. Data are presented as mean ± standard error. For RT-PCR experiments, $N\geq3$

undifferentiated hMSCs, $M=-5.684$ (fdr$=0.008$); osteogenic differentiation *versus* undifferentiated, $M=-2.434$ (fdr$=0.029$); adipogenic differentiation *versus* undifferentiated, $M=-1.141$ (fdr$=0.103$). After validating the microarray results by real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 1b), we selected miR-335 for further characterization.

miR-335 levels were also significantly reduced in human osteoblasts (Saos-2 cell line) after differentiation (Supplementary Figure S4).

hMSCs from different tissues express higher levels than fibroblasts of miR-335 and its host gene *MEST*. To determine whether relatively high expression of miR-335 is specific to bone marrow-derived hMSCs or is a common characteristic of hMSCs, we analyzed miR-335 expression levels in hMSC populations obtained from bone marrow, subcutaneous adipose tissue and articular cartilage (Figure 2b). All hMSC isolates were tested at passage 5–7 at the same cell density (8000 cells/cm^2), and were assayed for their differentiation potential to osteogenic, adipogenic and chondrogenic lineages in the presence of specific differentiation factors (data not shown). All three populations express significantly higher levels of miR-335 than dermal fibroblasts, and miR-335 expression levels were significantly higher in hMSCs isolated from bone marrow or articular cartilage than in hMSCs derived from adipose tissue.

miR-335 is encoded by the second intron of the *MEST* (mesoderm-specific transcript homolog) gene (Figure 2a).[16] *MEST* expression, determined by real-time RT-PCR, correlated with the levels of mature miR-335 (Figure 2b; Spearman's $r=0.5769$, $P=0.0008$), suggesting that the different hMSC isolates co-express mature miR-335 with its host gene and not through an *MEST*-independent mechanism (such as a specific promoter or post-transcriptional regulation).

MEST expression levels also correlated with the levels of miR-335 under all other conditions tested in this study (Supplementary Figure S5).

miR-335 impairs hMSC proliferation, migration and differentiation. We next analyzed the effect of miR-335 overexpression in bone marrow-derived hMSCs. hMSCs were transduced with the lentiviral vector pLV-EmGFP-MIR335, which encodes the genomic sequence spanning miR-335, or with a control vector (pLV-EmGFP-Mock). Transduced cells were purified to $>95\%$ homogeneity (gfp-positive cells) by fluorescence-activated cell sorting (FACS). To avoid non-specific effects due to lentiviral gene silencing or to a high proviral copy number per cell, a multiplicity of infection (MOI) of 5 was used, and only cells with medium-level gfp expression were selected (Supplementary Figure S3A). Real-time RT-PCR demonstrated an $\sim$3-fold increase in miR-335 expression in pLV-EmGFP-MIR335-transduced cells compared with controls (Supplementary Figure S3B).

When cultured over several passages, miR-335-overexpressing hMSCs showed a significant reduction in their proliferative activity compared with control cells (Figure 3a). However, miR-335 overexpression did not cause significant alterations to cell cycle kinetics (not shown) or the rate of apoptosis (Figure 3b).

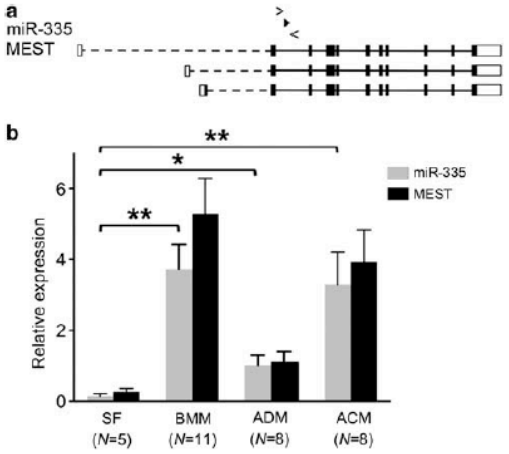

Figure 2 The expression levels of miR-335 in human mesenchymal cells correlate with those of its host coding-gene *MEST*. (**a**) Simplified map (5′→3′) of the human MEST locus (chromosome 7: 130 126 046–130 146 133) showing the three MEST transcript variants (lower line/box diagrams) and the position of the genomic sequence encoding miR-335 (MIRN335, upper black arrowhead). Boxes represent exons. Black boxes/solid lines indicate coding regions, and white boxes/dashed lines indicate untranslated regions. Arrowheads mark the position of the primers used to amplify the miR-335-encoding sequence for cloning into the lentiviral expression vector. (**b**) Relative expression levels of miR-335 (endogenous control RNU-48) and MEST (endogenous control GAPDH) were measured by real-time RT-PCR in human skin fibroblasts (SF) and in hMSCs isolated from bone marrow (BMM), adipose tissue (ADM) or articular cartilage (ACM). Data are means ± standard error. *$P\leq0.05$; **$P\leq0.005$ (Mann–Whitney *U*-test). The number of independent samples tested (*N*) is indicated on the *x* axis

hMSCs overexpressing miR-335 also showed an impaired migratory response to stimulation with fetal bovine serum (Figure 3c). Consistently, wild-type hMSCs transfected with an miR-335 inhibitor (Anti-miR-335, Ambion, Austin, TX, USA) showed increased migratory activity compared with cells transfected with a negative control Anti-miR oligonucleotide (Figure 3c; Supplementary Figure S3C). miR-335-overexpressing hMSCs also showed impaired migratory activity in an *in vitro* wound-healing assay compared with control cells (see Supplementary information videos). These results demonstrate that miR-335 is a negative regulator of hMSC migration.

The effect of miR-335 expression on hMSC differentiation capacity was monitored by comparing the *in vitro* differentiation responses of control and miR-335-transduced hMSCs to exposure to adipogenic or osteogenic stimuli for 3 weeks. Both adipogenic and osteogenic differentiation were significantly reduced by miR-335 overexpression. In particular, *in vitro* osteogenesis was almost completely abolished (Figure 3d), indicating a role for miR-335 in the regulation of hMSC differentiation programs. Analysis of expression of the differentiation markers osteopontin (osteogenic differentiation) and PPARγ (adipogenic differentiation) confirmed the inhibitory effect of miR-335 in both processes (Figure 3e).

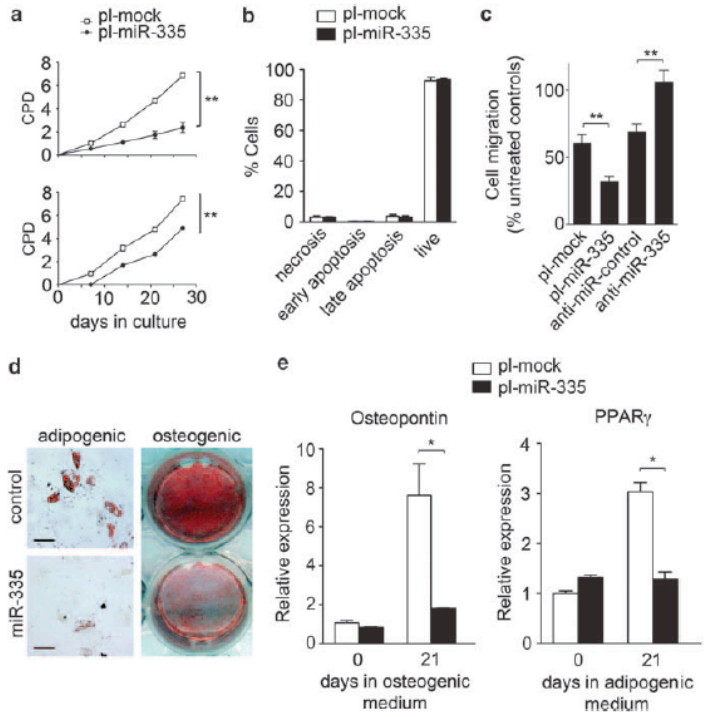

Figure 3 Exogenous miR-335 overexpression impairs hMSC proliferation, migration and differentiation. Bone marrow-derived hMSCs were transduced with the lentiviral vectors pLV-EmGFP-MIR335 or pLV-EmGFP-mock (encoding a negative control shRNA) and transduced (gfp +) cells were purified by FACS. The purified cells (pl-miR-335 and pl-mock, respectively) were used in gain-of-function studies. (**a**) Cumulative population doublings (CPDs) over several culture passages of pl-miR-335 (solid circles) and pl-mock (open squares) cells from two donors. *P*-values were calculated for CPD at the end of the assay. $N=3$. (**b**) Percent of living, necrotic and apoptotic cells determined by the Annexin V binding assay. Annexin V binding was analyzed by cell cytometry using Cell Quest software (BD Biosciences). $N=6$. (**c**) hMSCs (10^4) transduced with the indicated lentiviral vector or transfected with the indicated miRNA inhibitor were used in transwell migration assays. $N=3$. (**d**) hMSCs transduced with pLV-EmGFP-MIRN335 or pLV-EmGFP-mock (control) were cultured for 3 weeks in medium containing adipogenic or osteogenic factors, followed by staining with Oil Red O or Alizarin Red S, respectively. Representative images are shown. $N \geq 3$. Scale bars, 50 μm. (**e**) Relative expression levels of marker genes osteopontin and PPARγ (endogenous control GAPDH) were measured by real-time RT-PCR in hMSC cultured in osteogenic or adipogenic medium, respectively. $N=3$. For all panels, data are means ± standard error. $^*P \leq 0.05$; $^{**}P \leq 0.005$ (Mann–Whitney *U*-test)

***miR-335* expression levels are regulated by signals that control MSC migration and differentiation.** The apparent regulatory role of miR-335 in hMSC suggested that it might be regulated by signals known to control MSC biology. We therefore tested if canonical Wnt signaling, a negative regulator of MSC differentiation,[17] affected miR-335 expression levels. Bone marrow-derived hMSCs treated with 10 mM LiCl or Wnt3a-containing conditioned medium upregulated miR-335 expression (Figure 4a, left panel). Moreover, addition of Dkk1 (100 ng/ml), a specific inhibitor of canonical Wnt signaling, significantly decreased miR-335 expression in the presence of exogenous Wnt3a (Figure 4a, right panel). Upregulation of miR-335 expression by Wnt3a was also observed in adipose-derived hMSCs and in skin fibroblasts (Figure 4b), although in a different extension, suggesting that this effect is common to all mesenchymal cell types.

We next evaluated the effect on miR-335 expression of interferon-γ (IFN-γ) signaling, another key regulator of MSC activity. IFN-γ has numerous actions on MSC, including the induction of immunorregulatory activity, cell migration and osteogenic differentiation.[18,19] Treatment of bone marrow-derived hMSCs with human IFN-γ for 48 h significantly decreased expression of miR-335 (Figure 4c) in a dose-dependent manner, consistent with the lowered miR-335 expression during cell migration and osteogenic differentiation.

To identify possible binding sites in the *MEST* locus for transcription factors involved in β-catenin or IFNγ signaling pathways, we performed a multispecies (human, mouse and dog) sequence homology analysis for conserved binding sites for LEF/TCF (transcription factors involved in β-catenin signaling) and STAT1 (involved in IFNγ signaling). MULAN analysis with a matrix similarity of 0.95 showed one possible LEF1 and one possible TCF4 site in the 5-kb region upstream of the *MEST* transcriptional start site (corresponding to the longest human splicing variant), both mapping at −2675 kb (Supplementary Figure S6). A similar analysis, with a matrix

similarity of 0.90, identified three conserved potential STAT1-binding sites at −4440, −4384 and −4303 kb (Supplementary Figure S6).

Potential miR-335 targets in hMSCs are enriched in transcription regulators of cell movement and differentiation. In order to identify potential miR-335 targets, we used a two-step approach (Figure 5a). First, we employed three different miRNA target prediction programs, miRanda,[20] TargetScan[21] and PicTar,[22] to obtain a list of putative miR-335 targets. As these programs use different target scoring systems, we combined the three lists in a single non-redundant gene list (a total of 1838 genes, of which 1607 were mapped). Gene enrichment analysis using the PANTHER database revealed significant enrichment ($P<0.001$) for molecular functions related to transcriptional and post-transcriptional gene regulation, and for biological processes ($P<1E{-}06$) also related to regulatory activities, including mRNA transcription, protein modification and signal transduction (Supplementary Figure S7).

Second, we performed a microarray gene expression analysis (Agilent Whole Human Genome Microarray Kit) on hMSCs transduced with pLV-EmGFP-MIR335 or pLV-EmGFP-mock. We focused on downregulated genes since mRNA expression levels tend to correlate negatively with the expression levels of their specific miRNAs.[23] Using this approach, we identified 489 genes with decreased expression in miR-335-transduced cells that are thus candidate direct or indirect targets of this miRNA in hMSCs. Of these genes, 62 were also contained in the previously obtained list of predicted miR-335 targets (Supplementary Table S2), and were therefore classed as probable direct targets. This set of 62 genes was significantly enriched ($P<0.05$) for transcription factors (21%) and membrane-bound signaling molecules (6.5%). A detailed analysis with the Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, Redwood City, CA, USA, http://www.ingenuity.com) showed a predominant enrichment for functions involved in cell development, cell movement, gene expression and the cell cycle (Benjamini–Hochberg multiple test-corrected P-value = 1.37E 02–8.82E–02). Other significantly enriched biological functions included cell signaling, cell growth and proliferation, cell morphology, cell

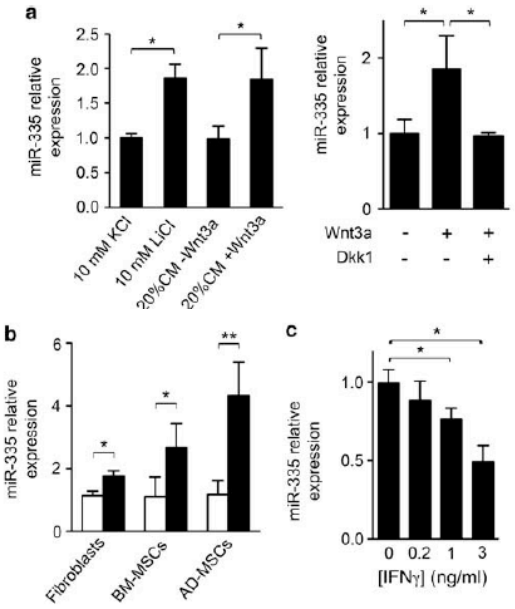

Figure 4 miR-335 expression is regulated by signals controlling hMSC proliferation, migration and differentiation. (**a**) Left panel, bone marrow-derived hMSCs (8000 cells/cm^2) were incubated for 48 h in the presence of medium containing 10 mM KCl, 10 mM LiCl, 20% conditioned medium without Wnt3a (CM–Wnt3a), or 20% Wnt3a-containing conditioned medium (CM + Wnt3a), and miR-335 relative expression was quantified by real-time RT-PCR. Right panel, relative expression of miR-335 in hMSCs cultured in 20% CM–Wnt3a, 20% CM + Wnt3a or 20% CM + Wnt3a containing 100 ng/ml of the Wnt inhibitor Dkk1. Relative expression levels are calculated as the fold change compared with the same cell type grown in standard culture conditions. (**b**) Human dermal fibroblasts (four different isolates), bone marrow-derived hMSCs (BM-MSC, two different isolates) and adipose-derived hMSC (AD-MSC, three different isolates) were incubated in 20% CM–Wnt3a (white bars), or 20% CM + Wnt3a (black bars), and miR-335 relative expression was quantified by real-time RT-PCR. Relative expression levels are calculated as the fold change compared with the same cell type grown in standard culture conditions. (**c**) Bone marrow-derived hMSCs were incubated with medium containing different concentrations of human IFNγ for 48 h, and miR-335 expression was determined by real-time RT-PCR. For all panels, data are means ± standard error. *$P\leq 0.05$; **$P\leq 0.005$ (Mann–Whitney U-test). $N\geq 3$

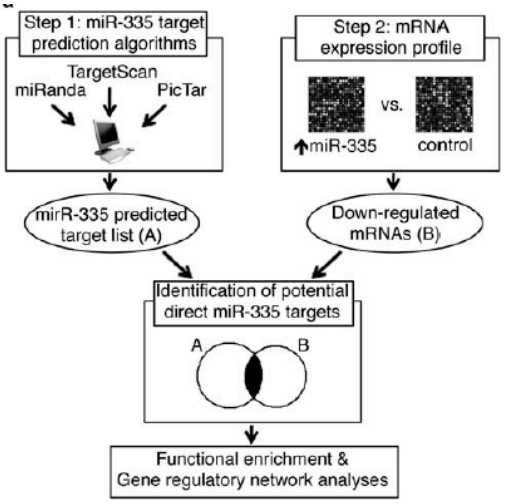

Figure 5 The set of potential miR-335 targets in hMSCs is enriched for cell movement and transcriptional regulatory functions. (**a**) Strategy used to identify potential mRNA targets of miR-335 in hMSCs. Sixty-two genes were identified by both target prediction algorithms and expression profiling (represented by the overlap in the Venn diagram). (**b**) Ingenuity Pathways Analysis was performed on the 20 cell movement-related genes and the 17 gene expression-related genes identified among the 62 potential miR-335 targets. The panels show the most significant networks found, including 11 (of 20, score 27) and 15 (of 17, score 42) genes, respectively (shaded)

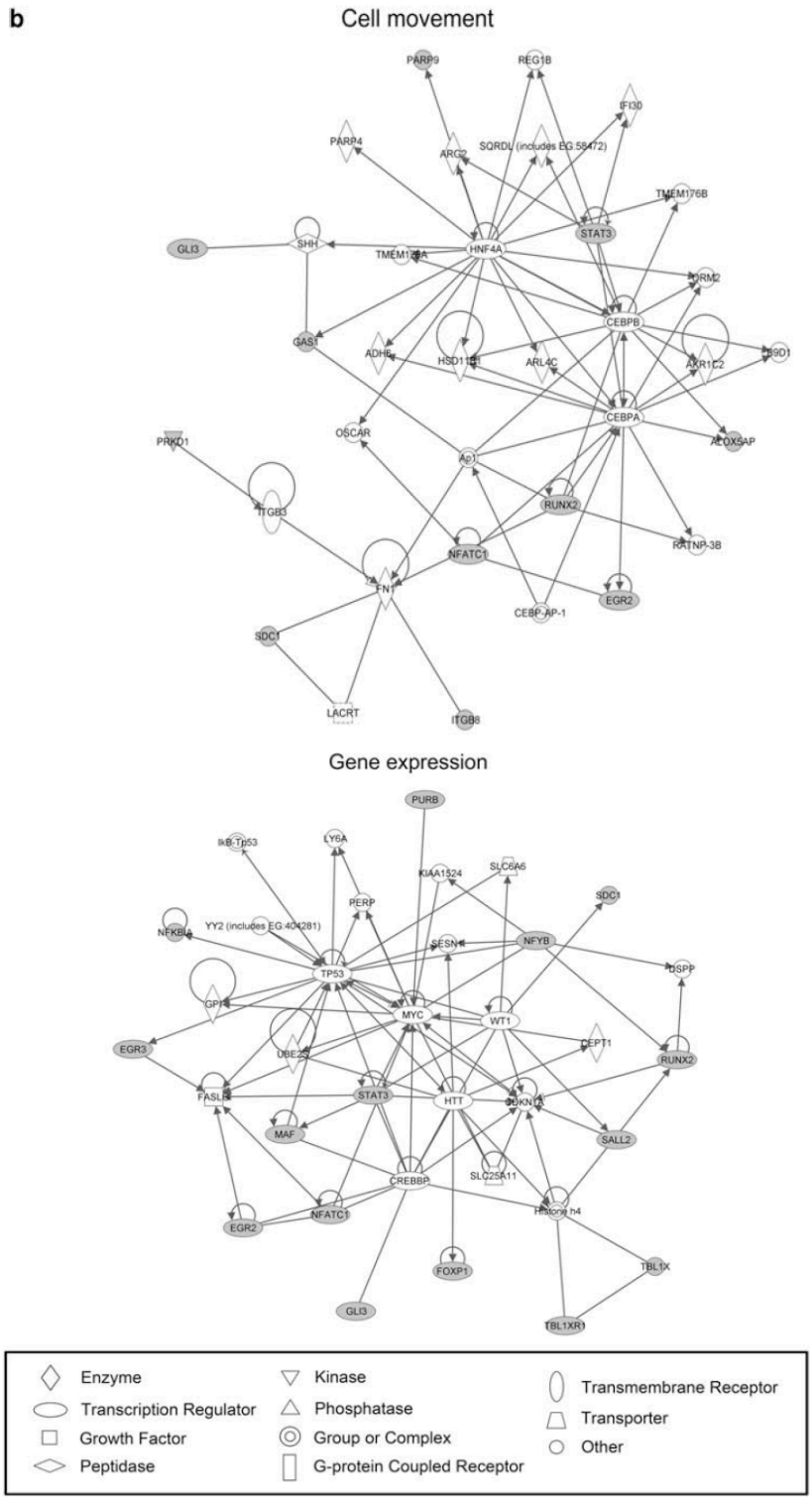

Figure 5 Continued

commitment and cell death (Table 1). Using IPA, the two more representative gene regulatory networks were constructed, respectively, containing 11 of the 20 potential miR-335 target genes involved in cellular movement (score 27) and 15 of the 17 genes involved in gene expression (score 42) (Figure 5b).

One of the predicted targets of miR-335 is RUNX2, a key transcription factor involved in osteogenesis. Although miRNAs can bind to coding regions and even to 5′regulatory regions, a single miR-335 binding site is predicted by the miRanda algorithm at nucleotides 3545–3567 in all the known *RUNX2* transcript variants (GenGank Accession Numbers NM_001024630, NM_001015051, NM_004348), corresponding to the 3′UTR region.

Table 1 Most significantly enriched molecular and cellular functions found using the IPA software in the list of 62 genes identified as potential miR-335 targets in hMSCs (B-H *P*-value: Benjamini–Hochberg multiple test-corrected *P*-value)

Relevant functions	B-H *P*-value	No. of Molecules
Cellular development	1.37E–02–8.82E–02	21
Cellular movement	1.37E–02–8.82E–02	20
Gene expression	1.37E–02–8.82E–02	17
Cell cycle	1.37E–02–8.82E–02	3
Cell signaling	1.76E–02–6.70E–02	4
Cellular growth and proliferation	2.47E–02–8.82E–02	19
Cell morphology	2.58E–02–8.52E–02	11
Cellular compromise	2.58E–02–5.84E–02	6
Cell death	2.58E–02–8.82E–02	18

A western blot analysis of RUNX2 demonstrated that its protein levels are reduced in hMSCs by miR-335 exogenous overexpression (Figure 6a). To determine whether miR-335 directly modulates RUNX2 expression levels in hMSCs, we cloned the full-length *RUNX2* 3′UTR downstream of the *Renilla* luciferase gene as a reporter, and assayed its expression in hMSCs transfected with a synthetic precursor or a specific inhibitor of miR-335. UTR reporters corresponding to *SOX4* and *UBE2F* genes were used as positive and negative controls, respectively, for the miR-335 inhibitory activity.[24] Transfection of the miR-335 synthetic precursor significantly reduced the expression of UTR reporters for *SOX4* and *RUNX2*, but not the expression of the negative control gene *UBE2F* (Figure 6b, upper panel). Transfection with the specific inhibitor of miR-335 significantly increased the expression levels of UTR reporters for *SOX4* and *RUNX2*, but did not affect the levels of *UBE2F* (Figure 6b, bottom panel). These results indicate that miR-335 controls RUNX2 expression in hMSCs by direct binding to its 3′UTR.

Finally, we have quantified the relative expression levels of *RUNX2* after osteogenic differentiation in hMSCs

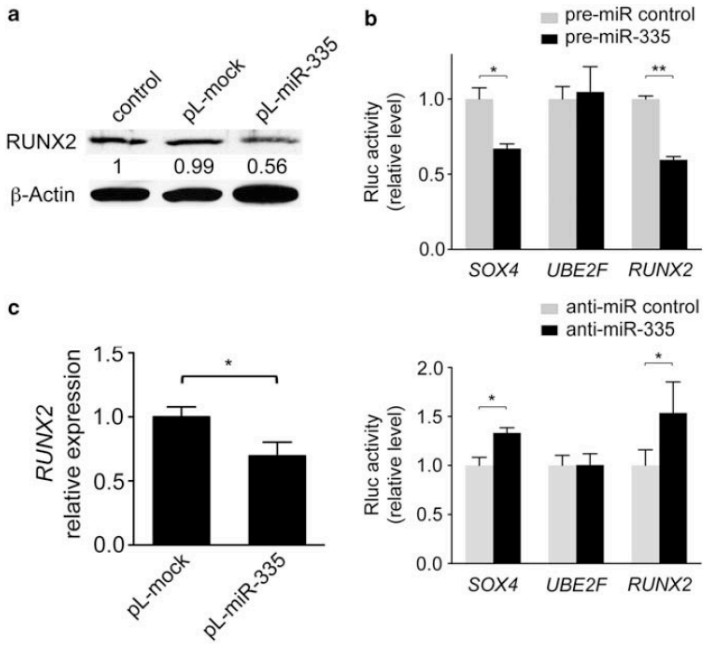

Figure 6 *RUNX2* is a direct target of miR-335. (**a**) Western blot of total cell protein (30 μg) from wild-type hMSCs and hMSCs transduced with a lentiviral vector encoding miR-335 (pL-miR-335) or a negative control shRNA (pL-mock). Numbers represent densitometry values of RUNX2 protein normalized to β-Actin standard. (**b**) UTR reporter assay of *RUNX2*. Reporter constructs consisting of the *Renilla* luciferase sequence fused to the 3′UTRs of *RUNX2*, *SOX4* (positive control) or *UBE2F* (negative control) were co-transfected with synthetic miRNA precursors or antagonists into hMSCs. *Renilla* luciferase (Rluc) activity was assayed 30 h after transfection, and the values were normalized to the activity of firefly luciferase encoded in the same vector. (Upper panel) Pre-miR-control (gray bars); pre-miR-335 (black bars). (Lower panel) Anti-miR-control (gray bars); anti-miR-335 (black bars). (**c**) Relative expression levels of *RUNX2* (endogenous control GAPDH) were measured by real-time RT-PCR in hMSC transduced with a lentiviral vector encoding miR-335 (pL-miR-335) or a negative control shRNA (pL-mock), cultured in osteogenic medium for 15 days. Data are means ± standard error. $^*P<0.05$; $^{**}P<0.005$ (Mann–Whitney *U*-test); $N=3$

overexpressing miR-335. The results of these experiments (Figure 6c) showed a significant reduction of *RUNX2* expression levels in the cells transduced with the lentiviral vector encoding miR-335, compared with those transduced with a control lentiviral vector, which is in good agreement with the rest of the data indicating that *RUNX2* is a target of miR-335 in hMSCs.

Discussion

Our results support a central role for miR-335 in the gene regulatory network that controls the tissue-repair activities of hMSCs. Expression of this miRNA is high in undifferentiated multipotent hMSCs compared with their differentiated cell progeny, and is regulated by major signaling pathways that control MSC biology, such as those controlled by Wnt3a and IFNγ. The set of predicted miR-335 targets is enriched in genes whose products are regulators of cellular movement and gene expression, including *RUNX2*, involved in the control of osteogenic differentiation.

There is growing evidence that miRNAs have a critical role in the biology of all stem cell types, including ES cells, germline stem cells and SSCs. As SSCs are usually less well defined than embryonic or germline stem cells, it has been difficult to identify the specific roles of an miRNA in a particular SSC population. Most miRNAs described as regulators of SSC biology are inducers of cell differentiation/commitment or inhibitors of self-renewal and proliferation, while others seem to be involved in the self-renewal or maintenance of some SSC types.[25]

Our results demonstrate that miR-335 is downregulated in hMSC cultures undergoing adipogenic or osteogenic differentiation, and is also expressed at comparatively lower levels in skin fibroblasts, which are mesenchymal cells with a limited differentiation potential. Our data are in complete accordance with previous work identifying miR-335 as the most upregulated miRNA in bone marrow-derived hMSCs in comparison with fibroblasts.[14]

Until recently, the only known specific biological role for miR-335 is in human breast cancer, where it has been proposed as a metastasis suppressor.[24] Expression of miR-335 is lost in the majority of primary breast tumors from patients who relapse, and this is associated with poor distal metastasis-free survival.[24] These results are in good agreement with our findings demonstrating that miR-335 is a negative regulator of hMSC migration. miR-335 downregulation is also observed in drug-resistant ovarian cancer cells,[26] cultured chondrocytes (*versus* cartilage tissue),[27] ethanol-treated neural progenitors,[28] multiple myeloma cells and tumors[29] and muscle regeneration.[30] In contrast, miR-335 is highly expressed in obesity models[31] and in fetal lung compared with adult tissue.[32] During the preparation of this manuscript, a communication describing the direct targeting of *RB1* by miR-335 has been published.[33] In this study, the authors establish the important role of this miRNA in the induction of p53-dependent cell cycle arrest after DNA damage. These results are in accordance with our findings demonstrating that miR-335 is a negative regulator of hMSC proliferation, and, taken together with our results, suggest an important role of miR-335/*RB1* activity in regulation/activation of hMSC tissue-repair activities.

miR-335 is encoded by the second intron of the maternally imprinted gene *MEST* (human chromosome 7q32).[16] Our results demonstrate that both miR-335 and *MEST* are coordinately upregulated in hMSCs from three different tissues (bone marrow, subcutaneous fat and articular cartilage) in comparison with skin fibroblasts. The biological relevance of this finding remains to be determined, but our results suggest that miR-335 expression levels could be used to discriminate between hMSCs and fibroblasts, or even between different MSC populations with diverse differentiation potentials.

We found no significant regulation of miRNAs previously described as regulators of osteogenic differentiation (miR-26a, miR-27a, miR-125b, miR-148b, miR-196a, miR-489 and miR-204/211) or adipogenic differentiation (miR-103, miR-107 and miR-143) under any of the conditions tested. This apparent discrepancy with published data might be related to the fact that our microarray analyses were performed on hMSCs at early differentiation stages, unlike other studies that used terminally differentiated MSCs. Also, the method we used for signal processing (quantile normalization)[15] probably contributed to an overall reduction in the variation of gene expression between samples.

The finding that miR-335 expression impairs the proliferative and migratory capacities of primary bone marrow-derived hMSCs and inhibits both adipogenic and osteogenic differentiation suggests that miR-335 is part of a common regulatory pathway for cell proliferation, migration and differentiation in MSCs. The existence of a partially overlapping regulatory circuitry for these biological processes has already been demonstrated in MSCs,[17] as well as in other SSC lineages.[34] The role of canonical Wnt signaling pathway (Wnt3a) as a positive regulator of miR-335 expression in hMSCs agrees with the evidence showing that Wnt3a inhibits osteogenic differentiation in MSCs.[17] However, the observed reduction in proliferation capacity in hMSCs overexpressing miR-335 suggests that the reported proliferative effect of Wnt3a acts via miR-335-independent mechanisms. Also, it has been reported that Wnt3a induces ECM invasion in hMSCs,[35] although it inhibits it in murine MSCs.[36] Further studies are needed to determine the effects of Wnt3a in the migration capacity of hMSCs in different regulatory conditions, and if they are related to miR-335 upregulation.

In contrast with the effect of Wnt3a, IFNγ decreased miR-335 expression in hMSCs. Considered together with the anti-migratory and differentiation effects of miR-335, this action might account, at least partially, for the previously described pro-migratory and pro-osteogenic activities of IFNγ.[19] This cytokine has a key role in the therapeutic activity of MSCs, as it is involved in the activation of their immunomodulatory phenotype.[18] IFNγ signaling, both intercellular and autocrine, might thus regulate an unsuspected large number of genes in MSCs through the inhibition of miR-335 expression. At this time, we do not know the precise molecular mechanism by which IFNγ downregulates miR-335 expression in MSC, but it might involve an indirect effect through its previously described inhibitory action on the canonical Wnt signaling pathway.[37]

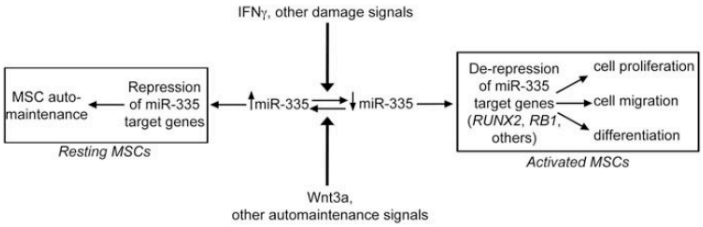

Figure 7 Working model for miR-335 control of MSC activation. In healthy tissues (homeostasis), physiological levels of Wnt3a, in co-ordination with other stimuli, keep miR-335 expression levels high in MSCs. Upon tissue damage or challenge, IFNγ, probably also in combination with other proinflammatory signals, induce miR-335 downregulation, which in turns causes the de-repression of its target genes, *RUNX2* among them. This activates the MSC reparative phenotype, characterized by an increased proliferative, migratory and differentiation capacities

Comparative genomics is a powerful new tool for identifying and characterizing functional sequences. The presence in the 5′ upstream region of the *MEST* locus of highly conserved potential LEF1 and TCF4-binding sites (95% similarity between human, mouse and dog) and potential STAT1-binding sites is compatible with the regulation of the *MEST*/miR-335 locus by both Wnt3a and IFNγ.

Consistent with our experimental results, the set of 62 potential direct gene targets of miR-335 is significantly enriched in genes related to cell movement and gene expression, pointing to a key regulatory role of miR-335 in MSC biology. In particular, we have experimentally validated RUNX2, a transcription factor essential for osteogenic differentiation, as a direct miR-335 target. Recently, miR-204 and miR-211 have been described as negative regulators of RUNX2.[12] Taken together, these results demonstrate that osteogenic differentiation of mesenchymal precursors is

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To our knowledge, this is the first demonstration of the involvement of a specific miRNA in the coordinated regulation of MSC proliferation, migration and differentiation. Our findings are summarized in the model presented in Figure 7. miR-335 is downregulated in MSCs in response to tissue damage signals, such as IFNγ, which leads to de-repression of miR-335 target genes involved in MSC proliferation, migration and osteogenic differentiation. These results have implications for the understanding of the major molecular mechanisms controlling the therapeutic activity of hMSCs *versus* their maintenance in an undifferentiated state, and strongly suggest an important role of miR-335 in tissue homeostasis.

Materials and Methods

Cell culture. This study was carried out according to the guidelines of the Instituto de Salud Carlos III (Spain). hMSCs from bone marrow, adipose tissue and articular cartilage were isolated and characterized as previously described.[38] Human skin fibroblasts were obtained from Inbiobank (San Sebastián, Spain). The 293T and the Saos-2 cell lines were purchased from The American Type Culture Collection (CRL-11268, Manasas, VA, USA). All cell lines were maintained in expansion medium, consisting of Dulbecco's modified Eagle's medium supplemented with 1 g/l D-glucose, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamicin; all culture reagents were from Invitrogen (Carlsbad, CA, USA). Cells were cultured in a humidified 37°C incubator at 5% CO_2.

For Wnt stimulation, cells were exposed to different concentrations of conditioned medium from Wnt3a-expressing QT6 cells. Control and Wnt3a-expressing QT6 cells were kindly provided by Dr. D Büscher (Cellerix SL, Madrid, Spain).

When indicated, several factors (DKK1, R&D Systems, Minneapolis, MN, USA; IFNγ, PeproTech, Rocky Hill, NJ, USA) or chemicals (KCl/LiCl, Sigma-Aldrich, St. Louis, MO, USA) were added to the hMSCs cells to evaluate its effect both on the modulation of miR-335 expression or on the several properties of the cells. In each case, the range of concentrations used is indicated.

Lentiviral vectors. The lentiviral vectors pLV-EmGFP-MIR335 (encoding the human miR-335 gene) and pLV-EmGFP-Mock (encoding a non-specific shRNA sequence) were based on pRRLsin18.PPT.CMV.GFP.Wpre, kindly provided by Dr. Luigi Naldini.[39] Detailed construction procedures for both plasmids are provided in Supplementary Information.

Lentiviral particles were produced by transient transfection of 293T cells.[39] Lentiviral transduction was carried out by overnight incubation of 3×10^5 hMSCs in 10 cm plates with 3 ml culture medium containing lentiviral particles at a MOI = 5. Transduced (gfp +) cells were purified 48 h after transduction by FACS using a FACSAriaII SORP cell sorter (BD Biosciences, Franklin Lakes, NJ, USA).

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Transfection of miRNA precursors and inhibitors. Anti-miR miRNA inhibitors (Ambion) are chemically modified, single-stranded nucleic acids designed to specifically bind and inhibit endogenous miRNAs. Pre-miR miRNA precursors (Ambion) are synthetic double-stranded oligoribonucleotides designed to match the sequence of specific miRNA:miRNA* duplexes. Depending on the experiment, Anti-miR or Pre-miR oligos at a final concentration of 50 nM were transfected (Lipofectamine 2000, Invitrogen) into 2×10^4 hMSCs cultured in 24-well plates (BD Biosciences) according to the oligonucleotide manufacturer's protocol.

Real-time RT-PCR. Total RNA was isolated from cultured cells with an miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Human miR-335, *MEST*, *SPP1* (osteopontin), *PPARG* and *RUNX2* transcripts were quantified by real-time RT-PCR using the corresponding TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). RNU48 and GAPDH were used as endogenous normalization controls for miRNAs and protein-coding genes, respectively.

Analysis of cell proliferation, cell cycle and apoptosis. Cell proliferation was assessed by seeding 6×10^4 cells/2 ml/well in six-well plates (Corning, Lowell, MA, USA) and culturing for 30 days. The medium was replaced every 3–4 days, and the cells were harvested and counted once a week. Cumulative population doubling (CPD) was calculated as $\log_{10}$(number of cells harvested)–$\log_{10}$(number of cells seeded)/$\log_{10}$.[2]

For cell cycle profiling, $\sim 1 \times 10^6$ methanol:acetic acid-fixed cells were incubated with 10 μM propidium iodide and 10 μM RNase A for 1 h at 37°C and analyzed by flow cytometry using a Becton-Dickinson LSR cytometer. Data were analyzed with the Summit v4.3 software (Dako Inc., Carpinteria, CA, USA).

For apoptosis analysis, cells were harvested and centrifuged at 2000 × *g* for 5 min at room temperature, washed twice with PBS, and resuspended in 1 × binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. A measure of 5 μl of Annexin V-DY634

(Immunostep, Salamanca, Spain) were added to 4×10^5 cells suspended in 400 μl of binding buffer. As a negative control, 2×10^5 cells were used without addition of Annexin V antibody. Cells were vortexed gently and incubated in the dark for 15 min at room temperature. Cells were centrifuged at 1000 $\times$ *g* for 5 min, and 1 $\times$ binding buffer (500 μl) and PI (5 μl) were added to each tube. The samples were analyzed by FACScan (BD Biosciences) using the Cell Quest software (BD Biosciences).

Cell differentiation assays. hMSCs were seeded at 2×10^4 cells/cm^2 in expansion medium, and after 24 h, medium was replaced by the corresponding induction medium. Osteogenic medium contained expansion medium supplemented with 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 0.2 mM ascorbic acid. Adipogenic medium contained expansion medium supplemented with 0.01 μM dexamethasone, 0.5 mM IBMX (3-isobutyl-1-methyl xanthine) and 60 μM indomethacin. In all cases, induction medium was replaced every 3–4 days and, on day 9 or 21 (depending on the experiment) cells were processed for histochemical analysis. Cells were fixed with 70% ethanol (1 h, 4°C) and stained (5 min). For osteogenic assays, cells were stained with 40 mM Alizarin Red, pH 4.1. Cells cultured in adipogenic medium were stained with 2% Oil Red O. Analysis of the expression of marker genes for osteogenic (osteopontin) and adipogenic (PPARγ) differentiation was performed by real-time RT-PCR as described above.

Cell migration assays. For transwell migration assays, hMSCs (1×10^4) were cultured in medium containing 0.2% FCS in a 24-well tissue culture insert with an 8-μm pore size membrane (BD Biosciences). Fresh culture medium containing 10% FCS was added to the bottom well. After 6 h, the filter membranes were fixed with 70% ethanol and mounted in mounting medium containing DAPI. Migration of hMSCs was determined by counting the number of DAPI-stained nuclei on the underside of the membrane under $\times$ 100 magnification, using CellProfiler image analysis software (http://www.cellprofiler.org).

For wound-healing assays, a 'wound' was created in a cell monolayer by scraping with a sterile plastic tip, and time-lapse video recordings were made by sequentially capturing microscopic images at 30 min intervals over a total period of 46 h.

Western blot analysis. Whole-cell lysates for western blotting were extracted with lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 and 0.25% sodium deoxycholate and protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). Protein samples were resolved by 10% SDS-PAGE and gels transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked with 5% non-fat milk for 1 h at room temperature and subsequently incubated overnight at 4°C with primary antibodies at the following dilutions: RUNX2, 1 : 500 (Sigma-Aldrich) and β-actin, 1 : 5000 (AbCam, Cambridge, MA, USA). Signals were detected using the appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark). The blots were visualized by an enhanced chemiluminescence system (ECL; GE Healthcare) according to the manufacturer's instructions, and relative intensity of the specific bands were quantified by densitometry using the ImageJ software (http://rsb.info.nih.gov/ij/).

UTR reporter assays. The full-length 3′UTRs of the putative miR-335 target gene *RUNX2*, as well as the negative control *UBE2F* and the positive control *SOX4* genes were amplified from human genomic DNA (Novagen, Madison, WI, USA) and individually cloned into the Psicheck-2 dual luciferase reporter vector (Promega, Madison, WI, USA). hMSCs were then co-transfected with each reporter construct and with an miRNA synthetic precursor (Pre-miR-335 or Pre-miR negative control #2, Ambion) or antagonist (Anti-miR-335 or at Anti-miR negative control #1, Ambion) at a final concentration of 50 nM, as described above. Cells were lysed 30 h after transfection and the ratio of *Renilla* to firefly luciferase was measured with the dual luciferase assay (Promega). Normalized *Renilla* to firefly ratios were determined in the presence or absence of miR-335 inhibition.

Microarray analyses. The Agilent Human microRNA Microarray v2.0 (G4470B, Agilent Technologies) was used to identify miRNAs highly expressed in undifferentiated hMSCs. miRNA expression profiles of undifferentiated hMSCs were compared with profiles of human skin fibroblasts and of the same hMSC lines after 9 days of adipogenic or osteogenic induction.

The Agilent Whole Human Genome Microarray Kit (G4112F, Agilent Technologies, Santa Clara, CA, USA) was used to identify genes downregulated in hMSCs exogenously overexpressing miR-335. The mRNA expression profile of hMSCs transduced with lentiviral vector pLV-EmGFP-MIR335 was compared with that of hMSCs transduced with the control vector pLV-EmGFP-Mock.

A full description of the experimental procedures, data processing and statistical analysis used for both types of microarrays is included in Supplementary information. All microarrays have been submitted to the Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo; accession number GSE19232.

Bioinformatic analysis. Computational miRNA target prediction analyses were carried out using the algorithms miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de).

Human, mouse and dog MEST loci were aligned, and the extent of DNA sequence homology was computed with the web-based program MULAN (http://mulan.dcode.org).[40] Using MULAN and multiTF (http://multitf.dcode.org) with the TRANSFAC professional V10.2 library database (http://www.biobase.de), binding sites for LEF1, TCF4 (0.95 matrix similarity) and STAT1 (0.9 matrix similarity) were predicted in the 5-kb upstream region of the human *MEST* locus.

Target gene functions were profiled with PANTHER software (http://www.pantherdb.org/tools) and Ingenuity Pathways Analysis software (http://www.ingenuity.com).

Statistical analysis. Statistical analyses of experimental data were conducted with Prism 3.0 (Graphpad Software Inc., San Diego, CA, USA). Unless otherwise stated, data were compared by the Mann–Whitney *U*-test for statistical significance. Results were considered statistically significant at $P \leq 0.05$.

Conflict of interest

The authors declare no conflict of interest.

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2. miR-335 correlaciona con la senescencia/envejecimiento de las células madre mesenquimales humanas e inhibe sus propiedades terapéuticas mediante la inhibición de la actividad de AP-1 (Tomé *et al.,* Stem Cells. 2014 Mar 19. doi: 10.1002/stem.1699. [Epub ahead of print]).

El análisis de la expresión de miR-335 en hMSCs provenientes de donantes de diferentes edades demostró una fuerte correlación directa entre los niveles endógenos de miR-335 y el envejecimiento. Además, la senescencia replicativa indujo un aumento significativo de la expresión de miR-335. Por el contrario, los factores capaces de frenar la senescencia celular, como la actividad de TERT y el cultivo en normoxia (3% de oxígeno), disminuyeron la expresión de miR-335. Las señales implicadas en la activación de las hMSCs (citoquinas y factores de crecimiento), también disminuyeron la expresión de este miRNA.

Por otro lado, y de forma inesperada, nuestro estudio demostró que la expresión forzada de miR-335 en hMSCs producía la aparición de un fenotipo senescente, caracterizado por un aumento en la actividad SA-β-gal, incremento de p16, así como una menor capacidad proliferativa, disminución de SOD2 y la aparición de un fenotipo secretor asociado a senescencia (SASP).

Además, la expresión exógena de miR-335 en hMSCs anuló tanto la capacidad de diferenciación osteocondral como la actividad antiinflamatoria de las hMSCs *in vivo*. Estos efectos fueron acompañados por una reducción severa en la capacidad de migración frente a numerosos estímulos. Mediante el uso de factores inductores de migración y diferenciación en hMSCs, observamos que la sobreexpresión de miR-335 causa la inhibición de la activación de AP-1, un factor de transcripción con un papel clave en la regulación de procesos como diferenciación, proliferación y migración. Al estudiar las proteínas implicadas en la activación de AP-1, encontramos que la sobreexpresión de miR-335 inhibe la fosforilación de la proteína quinasa D1 (PRKD1), crítica en la transmisión de señales extracelulares que conducen a dicha activación. La consecuente represión de AP-1 causó la incapacidad de respuesta de las hMSCs para adquirir su fenotipo reparador.

En este artículo proponemos que la regulación de miR-335 se relaciona con la senescencia y el envejecimiento de las hMSCs; niveles elevados de este miRNA son capaces de promover la aparición de un fenotipo senescente y disminuir drásticamente la

capacidad de estas células para responder a los diferentes estímulos que promueven su activación y efecto terapéutico.

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Mir-335 Correlates with Senescence/Aging in Human Mesenchymal Stem Cells and Inhibits their Therapeutic Actions through Inhibition of AP-1 Activity

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Key words. Mesenchymal stem cells • miRNA • Immunotherapy • Aging

ABSTRACT

MicroRNAs (miRNAs), small non-coding RNAs, regulate gene expression primarily at the posttranscriptional level. We previously found that miR-335 is critically involved in the regulation and differentiation capacity of human mesenchymal stem cells (hMSCs) *in vitro*. In this study, we investigated the significance of miR-335 for the therapeutic potential of hMSCs. Analysis of hMSCs in *ex vivo* culture demonstrated a significant and progressive increase in miR-335 that is prevented by telomerase. Expression levels of miR-335 were also positively correlated with donor age of hMSCs, and were increased by stimuli that induce cell senescence, such as γ-irradiation and standard O_2 concentration. Forced expression of miR-335 resulted in early senescence-like alterations in hMSCs, including: increased SA-β-gal activity and cell size, reduced cell proliferation capacity, augmented levels of p16 protein, and the development of a senescent-associated secretory phenotype (SASP). Furthermore, overexpression of miR-335 abolished the *in vivo* chondro-osseous potential of hMSCs, and disabled their immunomodulatory capacity in a murine experimental model of lethal endotoxemia. These effects were accompanied by a severely reduced capacity for cell migration in response to proinflammatory signals and a marked reduction in Protein Kinase D1 (PRKD1) phosphorylation, resulting in a pronounced decrease of AP-1 activity. Our results demonstrate that miR-335 plays a key role in the regulation of reparative activities of hMSCs and suggests that it might be considered a marker for the therapeutic potency of these cells in clinical applications. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Human mesenchymal stem cells (hMSCs) have become an important tool for cell-based therapeutic strategies. These cells can be easily isolated and expanded from the stroma of virtually all organs, although the preferred sources are bone marrow and subcutaneous fat. Experimental and clinical models have demonstrated conclusively that administration of *ex vivo*-expanded hMSCs can be effective in ameliorating some of the most prevalent clinical conditions which are currently poorly responsive to more conventional therapies (reviewed in [1]). *In vivo*, hMSCs are engaged in general tissue homeostasis, including cell proliferation, differentiation and migration, as well as regulation of angiogenesis and the immune response [2]. However, the molecular mechanisms which govern their biological and therapeutical activities remain unclear. Additionally, the

poorly-understood therapeutic properties of hMSCs are sensitive to culture conditions during *ex vivo* expansion, and are also negatively affected by donor age [3-6].

From extensive studies on primed differentiation of murine embryonic stem (ES) cells it was concluded that efficient maintenance of stem cells requires a highly coordinated regulation of gene expression [7, 8], involving both coding genes and noncoding RNAs (ncRNAs). Among the several regulatory elements involved in the regulation of stem cell function, microRNAs (miRNAs) play an important role. miRNAs are an abundant class of small ncRNAs that regulate the translation, stability and localization of target messenger RNAs; computational predictions of miRNA targets indicate that greater than 60% of all human protein-coding genes are regulated by miRNAs [9, 10]. Functional studies in ES cells have shown that miRNAs play essential roles, particularly in regulating the balance between self-renewal and differentiation [11, 12]. Less information is available on the role(s) of specific miRNAs in the regulation of MSC therapeutic activity; however, a number of relevant examples have been described, addressing areas from specific differentiation potential to hMSC aging (see Supplementary Table S1).

Using the same rational that allowed the dissection of self-renewal and differentiation mechanisms in ES cells, we attempted to identify miRNAs which are important for controlling the transition between the self-renewing (undifferentiated) and the reparative (differentiated) phenotypes in human bone marrow-derived MSCs. We found that miR-335 is the sole miRNA in hMSCs that is significantly downregulated in response to diverse differentiation stimuli [13]. In addition, miR-335 is the most highly upregulated miRNA in hMSCs in comparison with dermal fibroblasts, in agreement with previous data [14]. Up to that point, the only well-characterized description of miR-335 was its identification as a metastasis suppressor in human breast cancer cells [15]. We found that forced expression of miR-335 impairs the cell migratory capacity of primary bone marrow-derived hMSCs [13]. This finding has very interesting implications in view of our data showing that hMSC differentiation is associated with miR-335 downregulation. Indeed, we found that forced miR-335 expression also inhibits osteogenic and adipogenic differentiation of hMSCs *in vitro*. Thus, miR-335 is part of a common regulatory pathway for both cell migration and differentiation in hMSCs. The existence of such an overlapping regulatory circuitry for diverse biological processes has been demonstrated previously in MSCs, in addition to other cell lineages [16, 17]. Our data also established that miR-335 expression in hMSCs is regulated by the canonical WNT signaling pathway, which has been described as a regulator of MSC self-renewal, and by γ interferon, a pro-inflammatory cytokine that plays an important role in activating the immunomodulatory properties of hMSCs. We could associate all these effects with 62 putative targets of miR-335, which are strongly enriched for transcription regulators [13]. Taken together, these results strongly suggested that miR-335 downregulation could be a major trigger for the initiation of activities involved in tissue repair and remodeling, including cell migration and differentiation. However, the importance of miR-335 regulation for *in vivo* therapeutic activity of hMSCs, together with its possible role in immune regulation and its potential relationship with aging/senescence-related loss of reparative potential, remained to be addressed.

Here we demonstrate that both aging and prolonged *ex vivo* expansion of hMSCs, induces a progressive increase in miR-335 expression. Our results show that a relatively high level of miR-335 expression in hMSCs is associated with cell senescence alterations, and results in an essential loss of their therapeutic capacity. Mechanistically, this is linked to a significantly reduced capacity to activate protein kinase D1 (PRKD1), which in turn reduces the activity of the AP-1 transcription factor.

MATERIALS AND METHODS

Cell Culture

Bone marrow-derived hMSCs were obtained from Inbiobank Stem Cell Bank (http://www.inbiobank.org), and cultured in low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (1000 U/ml). All culture reagents were obtained from Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Cells were cultured at 37ºC in a humidified 5% CO_2/95% air atmosphere incubator and were passaged once per week, and media was changed twice weekly. Cell proliferation and SA-β-Gal activity were quantified as described in Supplementary Information. In some experiments, cells were γ-irradiated as described (Supplementary Information). The study was carried out in accordance with guidelines of the Instituto de Salud Carlos III (Madrid, Spain).

Lentiviral transduction

The lentiviral vectors pLV-EmGFP-MIR335, (encoding the human miR-335 gene) and pLV-EmGFP-Mock (encoding a non-specific shRNA sequence) were described previously [13]. The lentiviral vector encoding the telomerase reverse transcriptase catalytic subunit (pRRL.hTERT) has also been described [18].

Real-time quantitative PCR

Total RNA was isolated from cultured cells with the miRNeasy mini prep Kit (Qiagen, Valencia, CA, http://www1.qiagen.com). Transcripts of human miR-335, MAF, ATF3, JUN, JUNB, FOS, FOSB, COX2, IGF2, CXCL12, H19, PTGS2, TP53, CDKN1A, CDKN2A, AKAP9, RNASE2 and SCIN were quantified by real-time reverse transcription (RT)-PCR using the corresponding TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com).

RNU48 and GAPDH genes were used as endogenous normalization controls for miRNAs and protein-coding genes, respectively. For quantification of IL6 and IL8 expression, quantitative PCR was performed using the Roche Universal ProbeLibrary (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com) with α-tubulin as endogenous control, and the following primer-probe combinations: IL6 (Probe 45; F: GCCCAGCTATGAACTCCTTCT, R: GAAGGCAGCAGGCAACAC), IL8 (Probe 72; F: AGACAGCAGAGCACACAAGC, R: ATGGTTCCTTCCGGTGGT), and α-tubulin (Probe 58; F: CTTCGTCTCCGCCATCAG; R: TTGCCAATCTGGACACCA).

Nuclear Morphometric Analysis (NMA)

Analysis of nuclear morphometry was performed as described [19], using fixed cells permeabilized with 0.1% triton X-100, and labeled with 4⊡,6⊡-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma-Aldrich). The percentage of small and regular nuclei was determined as described [19].

Immunofluorescence

Cells were cultured on glass chamber slides, fixed with 4% paraformaldehyde (PFA) for 10 min, and permeabilized with 0.1% Triton in phosphate-buffered saline (PBS). Samples were blocked for 30 min at room temperature with 5% goat serum and incubated with the primary antibody against p16 (clone G175-405; BD-Pharmingen, San Diego, CA, http://www.pharmingen.com) or Lamin B1 (M-20; Santa Cruz Biotech, Santa Cruz, CA, http://www.scbt.com), and the appropriate secondary antibody conjugated with Cy3. Images were quantified using the CellProfiler™ software (http://www.cellprofiler.org).

Secretome analysis

Subconfluent cultures (10,000 cells/cm^2) were washed and incubated in serum-free DMEM for 24 h to generate conditioned medium (CM), which was collected and cells counted. CM was analyzed using a custom human 51-plex Luminexassay (Affymetrix, Santa Clara, CA, http://www.affymetrix.com), as described in Supplementary Information.

Western blot analysis

Whole-cell lysates for western blotting were prepared as described [4], and protein lysates were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Membranes were blocked with 5% non-fat milk for 1 h at room temperature and subsequently incubated overnight at 4°C with primary antibodies at the following dilutions: JUN, p-JUN, FOS, p-FOS, JUNB 1:500 (60A8, D47G9, 9F6, D82C12, C37F9, respectively; all from Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com); Mn-SOD2 1:500 (06-984; Millipore, Billerica, MA, http://www.millipore.com); p21 and p53 1:200 (sc-756, sc-99, respectively; Santa Cruz); β-actin 1:5000 (8226, Abcam, Cambridge, MA, http://www.abcam.com); p16 1:500 (554079; BD Pharmingen, San Jose, CA, http://www.bdbiosciences.com); PKD/PKCμ, Phospho-PKD/PKCμ (Ser916), Phospho-PKD/KCμ (Ser744/748) 1:1000 (2052, 2051, and 2054, respectively; Cell Signaling Technology), were used for SER 910 and 938/942 detection respectively; SRF 1:500 (NBP1-61263; Novus Biologicals, Littleton, CO, http://www.novusbio.com). Signals were detected using the appropriate peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark, http://www.dako.com). Western blots were scanned and quantified by densitometry using ImageJ software (NIH, Bethesda, MD, www.nih.gov).

Flow cytometry analysis of mitochondrial mass and reactive oxygen species (ROS) production

MSCs were detached with trypsin and resuspended in HBSS/Ca/Mg phenol-red-free medium (Sigma-Aldrich) at 10^6 cells/ml. Samples were incubated at 37°C in the dark with 5 μM dihydroethidium (DHE) red for 30 min, or with 1 μM MitoSOX™ Red, or 100 nM MitoTracker® Deep red probes for 30 min (all probes from Molecular Probes, Invitrogen). Cells were counter-stained with TOPRO-3 (for cells incubated with DHE or MitoSOX) or DAPI (for cells incubated with MitoTracker), to visualize the nuclei.

Diffusion chamber *in vivo* culture

Diffusion Chambers (DCs) were assembled from commercially available components (Millipore). Cell-loaded DCs (one per animal) were implanted subcutaneously into 8-week-old Fisher-344 rats. DCs were removed from the animals 4 weeks after implantation and fixed in 20% buffered formalin. The harvested chambers were decalcified in 4% EDTA solution, and then dehydrated with an ethanol gradient prior to embedding in paraffin wax. Standard histopathological analysis was performed on sections (5 μm) following hematoxylin and eosin staining, type I collagen, Sirius red (for collagen), mineralization by von Kossa, as well as Alcian blue and toluidine blue (for cartilage detection).

Lymphocyte proliferation assay

Buffy coat preparations were obtained from whole blood of healthy volunteers following the guidelines of the Centro de Transfusión de la Comunidad de Madrid, Spain. Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats by density sedimentation on Ficoll-Hypaque (Sigma-Aldrich) gradients (20 min, 2000 rpm, at room temperature). PBMCs (10^5) were cultured in duplicate with Roswell Park Memorial Institute (RPMI) complete medium in the presence of phytohemagglutinin (PHA, 10 μg/ml, Sigma-Aldrich) with or without various amounts of hMSCs (2x10^3 to 5x10^4) in flat-bottom 96-well plates. Prolifera-

tion was evaluated after 72 h culture by BrdU incorporation (Roche Applied Science).

Induction of endotoxemia and sepsis

Endotoxemia was induced in 7- to 10-week-old BALB/c male mice (Harlan Laboratories, Gannat, France) by intraperitoneal (i.p.) injection of 400 µg lipopolysaccharide (LPS)/mouse (*E. coli* serotype 055:B5; Sigma-Aldrich) as described [20]. All experiments were performed in accordance with institutional guidelines for the Care and Use of Laboratory Animals in Research, and were approved by CNIC.

Macrophage culture

Peritoneal exudate mouse cells were elicited in 8-week-old BALB/c male mice (Harlan Laboratories) by i.p. injection of 2 ml of 3% sterile sodium thioglycolate (Sigma-Aldrich). Cells were obtained 3 days later by peritoneal lavage with cold PBS, washed in cold RPMI medium and cultured in RPMI complete medium at 10^6 cells/ml. Macrophage monolayers (10^6 cells/well) were incubated with RPMI complete medium in the absence or presence of LPS (1 µg/ml), and hMSCs (1:5 hMSC:macrophage cell ratio). To determine the cell-contact dependence of the co-culture response, LPS-stimulated macrophages (10^5) were placed in the upper insert of a transwell system (0.8 µm pore, Corning Inc., Corning, NY, http://www.corning.com), and hMSCs ($2x10^5$) were placed in the lower well. Cell-free supernatants were collected after 24 h incubation, and cytokine levels were determined as described in Supplementary Information. For the generation of macrophage-CM, macrophages were cultured for 24 h at 80% confluence in RPMI complete medium containing LPS (1 µg/ml).

Cell migration assay

The *in vitro* migratory potential of hMSCs, was evaluated by transwell migration assays in response to the following stimuli: HMG1 (10 ng/mL), fMLP (10 nM), SDF1 (1 ng/mL), TNFα (2.5 µg/ml), PDGBB (0.1 mg/ml), and macrophage-conditioned medium (Mϕ-CM; diluted 2:3 in RPMI complete medium). hMSCs ($1.5x10^4$) were cultured in fresh medium without FBS in a 24-well tissue culture insert with an 8 µm pore size membrane (Corning). Inserts were placed on top of wells containing the different stimuli. After 6 h, (3 h for Mϕ-CM) filter membranes were washed with PBS, and non-migrated cells were removed from the upper side using a cotton swab. Finally, membranes were fixed with 4% PFA and mounted in mounting medium containing DAPI. Migration of hMSCs was calculated by counting the number of DAPI-stained nuclei on the underside of the membrane under X200 magnification using CellProfiler™ image analysis software.

Statistical and functional analysis

Statistical analysis of experimental data was performed with Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA, http://www.graphpad.com). All values are expressed as mean ± standard error of mice/experiment. Unless otherwise stated, differences between groups were assessed by double-tailed Student´s t-test. Survival curves were analyzed by the Mantel-Cox log-rank test. Results were considered statistically significant at $p<0.05$. Functional gene analysis was generated as described in Supplementary Information.

RESULTS

miR-335 expression is associated with hMSC aging

Our previous studies identified miR-335 as the only miRNA consistently downregulated in hMSCs in all differentiation regimes tested, and its forced expression in hMSCs reduced their proliferation and differentiation capacity [13]. To further examine the regulation of miR-335 expression, we subjected a panel of bone marrow-derived naïve hMSCs to different environmental stimuli associated with the promotion of senescence, and measured expression levels by qRT-PCR. Compared with early passage cells, prolonged culture of hMSCs (>7 weeks) resulted in a significant increase in miR-335 expression (3.4-10.2 fold) in all hMSCs tested (Fig. 1A). miR-335 expression was also dependent on the oxygen tension used during culture, with mild hypoxia (3% O_2, for 7 days) significantly decreasing the level of miR-335 (Fig. 1B). Furthermore, expression of miR-335 was transiently increased by γ-irradiation (0.5-10 Gy, Fig. 1C); while stimulation of hMSCs with a range of pro-inflammatory growth factors resulted in a significant decrease of expression (Fig. 1D). Interestingly, miR-335 expression was also dependent on the donor age of bone marrow-derived hMSCs (Fig. 1E), and we found a strong positive correlation between the level of miR-335 and donor age from 15-55 years (Fig. 1E; Pearson's $r=0.9205$, $p=0.0033$). This result prompted us to examine the possible effect of telomerase for expression of miR-335. Telomerase activity prevents cells dividing recursively from reaching their Hayflick limit [21] and has been inversely correlated with aging [22, 23]. Results demonstrated that the increase in miR-335 expression during *ex vivo* culture was abolished by forced expression of TERT (Fig. 1F). Collectively, these findings indicated that an increase in miR-335 expression was associated with hMSC aging/senescence and could be prevented by telomerase expression.

Forced expression of miR-335 induces senescence-related changes in hMSCs

Given the positive correlation between miR-335 expression and senescence/aging, we wondered whether forced overexpression of miR-335 could induce a senescence/aging phenotype in hMSCs. To consider this, independent hMSCs were transduced with a lentiviral vector encoding the genomic sequence spanning miR-335 (335-hMSC), or with a control vector (control-

hMSCs), and transduced cells were purified to >95% homogeneity (GFP-positive cells) by FACS [13]. Comparative analyses of control- and 335-hMSCs in culture demonstrated that miR-335 overexpression resulted in manifold changes associated with cell senescence, including: reduced proliferation (Fig. 2A), morphological alterations (greater cell size, more vacuoles and an increased nuclear irregularity index; Fig. 2B), and a larger proportion of SA-β-gal positive cells (Fig. 2C). Furthermore, 335-hMSCs exhibited reduced expression of Lamin B1 (Fig. 2D), a protein lost in senescent cells [24]. In contrast, no significant differences were found in the cell cycle between control and miR-335-overexpressing cells (Supplementary Fig. S1), and genetic stability was comparable between both cell types, as monitored by karyotype analysis and comparative genomic hybridization (data not shown).

Guided by these results, we next analyzed the expression of candidate genes that have been previously associated with replicative senescence. These genes included TP53 [25], CDKN1A, CDKN2A [26], PTGS2 [27], and six genes that we have recently defined as markers of replicative senescence in hMSCs (SCIN, EDN1, AKAP9, CXCL12 [4], H19 and IGF2 (García *et al.*, submitted). Compared with control cells, 335-hMSCs showed a statistically significant increase in the expression of SCIN, and a decrease in IGF2, CXCL12 and H19 (Fig. 2E), supporting a pro-senescence activity of miR-335. In contrast, miR-335 overexpression resulted in a significant decrease in TP53 (p53) and CDKN1A (p21) mRNA, and also protein (Fig. 2E and 2F, respectively). Notably, the clear reduction of PTGS2 (COX2) expression observed in 335-hMSCs (Fig. 2E) is contrary to that previously observed in aged cells [27]. Interestingly, western blot analysis of 335-hMSCs also revealed a reduction in the steady-state levels of SOD2 (Fig. 2F), a protein whose deficiency has been recently linked to cellular senescence [28]. Moreover, p16 and cyclin D1 (CCND1), which are known to be associated with cellular senescence [26], were significantly increased in 335-hMSCs (Fig. 2F), and immunofluorescence analysis confirmed this increase in p16 expression (Fig. 2G). Taken together, these data clearly suggested that increased expression of miR-335 could favor the evolution of hMSC senescence through a p53/p21-independent mechanism.

miR-335 overexpression induces a senescence-associated secretory phenotype in hMSCs

Previous work demonstrated that fibroblasts undergoing senescence growth arrest secrete myriad factors, including proteins associated with inflammation, and develop a senescence-associated secretory phenotype (SASP) [29]. To determine whether overexpression of miR-335 induced a similar senescence-related phenotype, we compared the secretome of 335-hMSCs and control cells using conditioned medium (CM) from confluent cells. CM was analyzed with Luminex antibody arrays designed to detect 51 secreted human proteins involved in intercellular signaling during inflammation (Supplementary Table S2). Results indicated a significant (t-test, $p<0.05$) increase in 12 out of 51 tested factors in CM from 335-hMSCs compared with control cells, with ratios of oversecretion between 2-3 fold (Fig. 3A). Those identified proteins showed upregulated levels (normalized to 10^5 cells/ml) between 0.89 pg/ml (IFNγ) and 300.23 pg/ml (IL6) in CM from 335-hMSCs (Fig. 3A). Comparable results were obtained in hMSCs made senescent by γ irradiation, although fewer proteins were differentially oversecreted in 335-hMSCs (23.5%, compared to 52.9% in γ-irradiated hMSCs; [30]). The differential secretome of 335-hMSCs was similar to results obtained from a previous characterization of SASP in senescent human primary fibroblasts [29]. Additionally, RT-PCR analysis of the key SASP factors, IL-6 and IL-8, confirmed previous observations [29] and indicated that proteins comprising SASP are generally upregulated at the mRNA level (Fig. 3B).

When CM from control and 335-hMSCs were obtained in the presence of 1 mg/ml LPS, to mimic a proinflammatory condition, only GROA and HGF were significantly oversecreted in 335-hMSCs (Fig. 3C). The immune system processes (Gene Ontology, GO terms) associated to all the identified upregulated proteins are shown in Supplementary Table S3.

miR-335 overexpression induces mitochondrial changes in hMSCs

Alterations in both mitochondrial content and function are associated with senescence [31]. To determine whether this phenotype was also discernible in hMSCs, we performed mitochondrial staining in 335- and control-hMSCs using MitoTracker (MT), a marker of mitochondrial mass that is independent of membrane potential [32]. While the pattern of mitochondrial staining was comparable between 335- and control-hMSCs (data not shown), FACS analysis demonstrated a significant increase in mitochondrial mass in 335-hMSCs (Fig. 4A). Furthermore, analysis with MitoSOX Red, a dye which specifically detects superoxide within the mitochondrial matrix, revealed a significant increase in superoxide in 335-hMSCs compared with control cells (fluorescence increase approximately 3-fold; p=0,013; Fig. 4A), and this increase remained significant when expressed per unit of mitochondrial mass (Fig. 4B; a representative example is shown in Fig. 4C). This finding was consistent with the reduction in SOD2 expression observed in 335-hMSCs (Fig. 2F). Additionally, an increase in the relative levels of both total and mitochondrial superoxide was detected in 335-hMSCs stained with dihydroethidium (Fig. 4A). Together, these findings indicated that forced expression of miR-335 in hMSCs induced a significant increase in mitochondrial ROS production, which is presumably compensated by an increase in mitochondrial biogenesis.

Forced expression of miR-335 abrogates the *in vivo* chondro-osseous differentiation capacity of hMSCs

Previously, we demonstrated that forced expression of miR-335 in hMSCs strongly reduced their osteogenic differentiation potential *in vitro* [13]. To connect these observations with what happens *in vivo*, we utilized cell diffusion chambers in a rat model of osteogenesis. Control- and 335-hMSCs were first cultured in a collagen-gel medium containing 0.5% fetal bovine serum (FBS) for 10 days in the presence of recombinant human BMP2, followed by culture for 6 days in medium containing BMP2 and 10% FBS. This treatment has been described to be effective in initiating a chondro-osseous differentiation pathway in bone marrow hMSCs cultured *in vitro* [33]. After this 16-day period of differentiation, cells were placed inside diffusion chambers and implanted subdermically into the back of 8-week-old rats for 28 days. Whereas histological and immunohistochemical analyses provided clear evidence of collagen (Fig. 5A, panel 1-3; 6-9) cartilage (panel 6-9) and osteoid tissue (panel 4-9) inside the diffusion chambers seeded with control hMSCs, chambers seeded with 335-hMSCs failed to show any evidence of cartilage or osteoid tissue formation, and instead their content appeared as a mass of fibrous tissue (Fig. 5B). This complete abrogation of *in vivo* osteogenic potential demonstrated that miR-335 downregulation is physiologically necessary for bone differentiation of hMSCs.

miR-335 is critical in modulating the immunoregulatory capacity of hMSCs

Given the established immunomodulatory properties of MSCs, we next investigated whether overexpression of miR-335 in hMSCs altered their capacity to regulate the immune response. Using co-culture assays, we first assessed the potential of hMSCs to inhibit cell proliferation of human PBMCs stimulated with phytohemagglutinin. Compared with control cells, 335-hMSCs displayed a reduced, but still significant, capacity to inhibit PBMC proliferation measured by BrdU incorporation, after 72 h culture (Fig. 6A, 30.8% *vs.* 47.9% inhibition at a 1:5 ratio). As hMSC administration has a strong therapeutic effect in experimental sepsis, by protecting against mortality caused by endotoxin [34, 35], we next investigated whether forced expression of miR-335 could influence mortality in a LPS-induced endotoxemia model. Thus, mice were injected with 400 µg of LPS, together with wild-type hMSCs, control-hMSCs or 335-hMSCs (10^6 cells/mouse), and mortality was monitored over 96 h. As expected, administration of wild-type or control-hMSCs rescued the majority of animals from LPS-induced lethality (Fig. 6B). In contrast, inoculation of an equivalent number of 335-hMSCs did not provide any significant protection against LPS, and mortality was comparable to the administration of vehicle (Fig. 6B).

hMSCs are known to promote the conversion of macrophages to a regulatory (M2) phenotype through the secretion of soluble factors [36-38]. To evaluate the potential modulation of macrophage activity by miR-335 overexpression, we utilized co-culture assays of hMSCs and macrophages, and measured the concentration of cytokines released 24 h after macrophage activation with LPS. Compared with macrophage-only cultures, results showed that TNFα and IL-6 were decreased to similar levels in co-cultures of both 335-hMSCs and control-hMSCs with activated macrophages (Fig. 6C). Additionally, IL-10 was increased in both co-culture systems in comparison with macrophage-only cells, but only if there was direct cell-to cell contact as described in previous studies [35]. These results suggest that macrophage modulation is not a major component of the immunoregulatory capacity of hMSCs by miR-335.

Transwell migration assays revealed that overexpression of miR-335 caused a consistent reduction in the migratory capacity of hMSCs tested with different specific stimuli (HMG-1, 8.1%; fMLP, 8.5%; SDF1, 6.5%; TNFα, 4.7%; and PDGFBB, 14.5%) and also with LPS-stimulated macrophage-conditioned medium (Mϕ-CM) (25.4% compared to control; Fig. 6D). Consistent with this reduction in cell motility, 335-hMSCs expressed lower membrane levels of CXCR4 (Fig. 6E) the receptor for CXCL12 (SDF-1), which has been demonstrated to be an important player in hMSC migration *in vivo* [39, 40].

One of the major regulatory factors involved in macrophage reprogramming by hMSCs is prostaglandin E2 (PGE2). PGE2 levels depend on the activity of COX-2 (PTGS2), an enzyme induced in MSCs after stimulation with activated macrophages [35]. As described earlier, PTGS2 expression was downregulated in 335-hMSCs relative to control cells in non-stimulated conditions (Fig. 2B). Stimulation of 335-hMSCs with Mϕ-CM containing LPS also resulted in a marked decrease in the induction of PTGS2 (Fig. 6F), suggesting that PTGS2 could be an important mediator for the regulation of the hMSC anti-inflammatory response by miR-335. Interestingly, a detailed analysis of the microarray expression data obtained for 335-hMSCs [13], using the Ingenuity Pathway Analysis tool, generated a network (score 24) including 26 of the regulated mRNAs which have PTGS2 as nodal molecule (Supplementary Fig. S2).

Downstream mediators of miR-335 function in hMSCs

Analyses of previously obtained differential gene expression data [13], revealed that most members of the AP-1 family of transcription factors (including MAF, ATF3, JUN, JUNB, FOS and FOSB) were significantly downregulated in 335-hMSCs (Supplementary Fig. S3). Notably, AP-1 mediates the response to a variety of extracellular stimuli and plays a key role in the regulation of diverse processes such as differentiation, proliferation and migration (reviewed in [41]). A

downregulation of AP-1 components in three independent miR-335 expressing hMSC samples, measured under basal conditions, was detected by qRT-PCR, with the exception of ATF3 (Fig. 7A), and confirmed the previous microarray data [13]. Therefore, we hypothesized that regulation of the AP-1 complex by miR-3335 could be involved in both the control of hMSC migration and differentiation. When we measured expression of AP-1 components after stimulation of control- and 335-hMSCs with Mϕ-CM (used as a migratory stimuli, Fig. 6D), or BMP2 (used for chondro-osseous differentiation, Fig. 5), we found that under both conditions 335-hMSCs expressed significantly less JUN, JUNB, FOS, FOSB, ATF3 and MAF mRNA, and this was particularly evident with BMP2 stimulation (Fig. 7B). Western blot analysis confirmed the blunted expression of JUN protein in 335-MSCs after Mϕ-CM and BMP2 induction (Fig. 7C). Strikingly, both treatments resulted in an almost complete loss of FOS and p-FOS induction in 335-hMSCs compared with control cells (Fig. 7C). Since neither FOS nor JUN mRNA contain a miR-335 binding site, it seemed likely that AP-1 activity is regulated through an indirect mechanism. To address this possibility, we selected putative miR-335 targets that could have a direct effect on AP-1 activity [13], or an indirect impact on the MAPK kinase pathway leading to AP-1 activation (reviewed in [42]). These included Serum response factor (SRF), a transcription factor involved in FOS regulation [43], and Protein kinase D1 (PRKD1), a key regulator of MAPK activation [44, 45]. Thus, hMSCs were stimulated with Mϕ-CM, and the expression of SRF and PKD1 were measured by immunoblotting. Compared with control hMSCs, MΦ-CM-stimulated 335-hMSCs had a moderate reduction in SRF protein (Fig. 7D). On the other hand, whereas total PRKD1 protein did not differ significantly between groups, phosphorylation on residues Ser738/742 and Ser910 of PRKD1 was decreased markedly (greater than 60%) in 335-hMSCs compared with control cells, after stimulation with Mϕ-CM. Notably, PRKD1 plays a pleiotropic role in the regulation of many cellular processes, including cell migration and differentiation [46, 47]. Accordingly, reduction in the phosphorylation of critical regulatory residues in PRDK1 could reduce MAPK activation, and impact on downstream signaling cascades, including AP-1. In turn, a reduction in the activity of the AP-1 complex could abrogate the migration and differentiation characteristics of MSCs.

DISCUSSION

We previously identified miR-335 as being uniquely downregulated in hMSCs early after induction of differentiation, and its forced expression negatively impacted proliferation, migration and differentiation of hMSCs *in vitro* [13]. These results led us to conclude that miR-335 was important for the maintenance of the undifferentiated state of hMSC and/or it must be downregulated to allow differentiation in this cell type. These features might conceivably explain its original description as a tumor suppressor [15, 48].

It is well established that, due to oxidative stress, primary cells maintained in culture evolve towards a state of senescence/aging, which in turn leads to a progressive loss of their biological activity [3, 49]. Analysis of hMSCs revealed a clear increase in the expression of miR-335 during extended periods of *ex vivo* culture. This finding, together with the observation that telomerase overexpression downregulates miR335, argued for a genuine role for miR-335 in the promotion of senescence/aging, and is in agreement with its negative impact on hMSC function [13]. Here we demonstrate that forced expression of miR-335 abolishes chondro-osteogenic differentiation of hMSCs *in vivo*, and also significantly reduces their immunoregulatory properties *in vivo*. A very recent study has reported that miR-335 promotes chondrogenic differentiation in murine MSCs [50]. The discrepancy between these recent findings and those of this study might be due to a species-specific mechanism, or perhaps the different differentiation models used. The negative effects of miR-335 overexpression for both differentiation and immunoregulation of hMSCs can be at least partly explained by a decrease in their migratory capacity, together with a reduction in the expression of the migration receptor CXCR4. The fact that 335-hMSCs appear to maintain most of their intrinsic modulatory activity on macrophages suggest that the loss of their *in vivo* immunoregulatory activity is more likely due to their reduced migratory potential, rather than a defect in their signaling apparatus. These results are similar to those previously obtained by us in radiation-induced senescent hMSCs [30].

Interestingly, 335-hMSCs have lower basal levels of PTGS2 (COX-2), and fail to upregulate this gene in response to several stimuli. Of note, PTGS2 has been proposed to contribute to the establishment of the senescence phenotype and is known to be upregulated during both replicative and stress-induced senescence of cultured fibroblasts [27, 51, 52], and also during organismal aging [53-57]. However, it remains unclear whether the catalytic activity of PTGS2 is involved in the aging process [51], and paradoxically, constitutive overexpression of PTGS2 has been linked with cellular resistance to senescence in cancer cells [58]. Moreover, PTGS2 has been described as a key player in the immune regulatory activity of hMSCs and its loss appears to be a hallmark of the onset of cell senescence in this cell type [38].

In agreement with our findings, previous work has demonstrated an upregulation of both miR-335 and miR-34a in old (24-months-old), but not young (3-months-old), rat mesanglial kidney cells. In this model, senescence was mediated principally through inhibition of Sod2 and Txnrd2 by miR-335 and miR-34 respectively, which dramatically affected the mitochondrial antioxidant capacity of these cells [59]. Our data in 335-hMSCs also demonstrated a significant reduction in

SOD2 expression, together with enhanced superoxide production by mitochondria; both are hallmarks of cellular senescence and aging [28]. It remains to be determined whether the observed increase in mitochondrial content is due to a deregulation of mitochondria biogenesis, or is simply an adaptation to the overall increased cellular size of 335-hMSCs. Nevertheless, these data strongly suggest that miR335 is regulating the progression of senescence.

A balance between cell growth and response to stress must exist to ensure that cells proliferate without accumulating damaged DNA. Consequently, optimal cell proliferation requires the coordination of pro-growth and stress-response pathways. While cellular senescence is considered a cellular stress response, and widely recognized as a potent tumor suppressive mechanism, it has also recently been acknowledged as a partner in degenerative and hyperplastic pathologies, most likely by promoting chronic inflammation and aging [60]. Senescent cells have been shown to exhibit a senescence-associated secretory phenotype (SASP) that can alter the tissue microenvironment and promote age-related pathology through secretion of growth factors and proteases [29]. SASP is a damage response, separable from growth arrest [61, 62], and variable with cell type and mode of senescence induction. In an effort to decode the molecular mechanisms influenced by miR-335, we compared the secretome of 335-MSCs with control-MSCs. As described for other cell types [29], we did not detect a selective response in 335-hMSCs; however, the secretion of more than the 20% of the analyzed factors was increased 2-3-fold. The majority of these factors have been previously characterized as part of SASP in fibroblasts, but with some specific differences likely related to the cell type [29, 63]. These results are therefore consistent with the association of miR-335 with senescence promotion.

An important finding in this study was the significant relationship between miR-335 and AP-1 activity. Members of the FOS family dimerize with JUN to form the AP-1 transcription factor, which upregulates the transcription of a diverse collection of genes [64]. Moreover, AP-1 activation is involved in the regulation of cell migration [65]. Whereas control hMSCs responded to BMP2 or Mϕ-CM treatments by increasing their expression of JUN, mainly at the level of mRNA, this was blunted in 335-hMSCs, particularly when stimulated with BMP2. However, greatest differences between cells were found in the protein expression levels of FOS and p-FOS, and miR-335 overexpression almost completely abrogated FOS expression (Fig. 7). FOS is upregulated in response to many extracellular signals, and phosphorylation by MAPK, PKA, PKC or CDK1 alters its activity and stability. Interestingly, hyporesponsiveness to growth factors is a fundamental feature of cellular senescence and, in several models including senescent human diploid fibroblasts and aged mice, a proportional reduction in the expression of Fos has been demonstrated [66]. Therefore, the impact of forced miR-335 expression on the level of active FOS could play an important role in senescence promotion.

As neither FOS nor JUN mRNAs harbor canonical target sequences for miR-335, we reasoned that their modulation must be regulated indirectly. From previous bioinformatic analyses, we selected a panel of predicted targets of miR-335 which were plausible upstream regulators of FOS/JUN. Among them, we demonstrated that SRF was moderately decreased by overexpression of miR-335, whereas PRKD1 was severely infra-phosphorylated, both in basal and activated conditions.

SRF stimulates both cell proliferation and differentiation, and through binding to canonical serum response elements (SRE), regulates the activity of many immediate-early genes such as FOS, which is a downstream target of many pathways including vascular development [67] and heart fibrosis [68]. Information on the specific role of SRF in hMSC biology or senescence is limited; however, SRF knockdown abolished expression of αSMA induced by several differentiation stimuli in adipose tissue-derived hMSCs [69]. All these findings strongly suggest that even a moderate reduction of SRF expression could play a relevant role in miR-335-mediated induction of senescence in hMSCs.

PRKD1 has been proposed to act as a signal-regulated scaffold, integrating an assortment of different stimuli and influencing the subcellular localization of important partners. Phosphorylation of serines 910 and 738/742 by PKC, and their subsequent autophosphorylation, are associated with upregulated PRKD1 activity [70]. Besides these central regulatory modifications, additional phosphorylation of PRKD1 by p38, Src and c-Abl kinases have also been described to modulate its activity, as well as cleaveage by caspase-3 during apoptosis [70]. PRDK1 activation by DAPK1 has been implicated in the induction of autophagy in response to oxidative damage [71]. Hitherto, no specific role for PKRD1 has been described in hMSC biology or cellular senescence. Our results suggest that reduced activation of PRKD1 by miR-335 could result in severe reduction of AP-1 activity, although the precise mechanisms involved requires further study.

Collectively, our results propose a scenario whereby miR-335 promotes the evolution of hMSCs towards a senescent stage by reducing the activity of AP-1. In addition to oxidative damage [72] a decline in fos/AP-1 activity is typically associated with aging; basal levels appear similar in young and old animals but in the latter the capacity to modulate fos/AP-1 activity is greatly impaired [72]. A reduction of AP-1 expression seems likely to be related to cell senescence also, since JNK-deficient cells exhibit early p53-dependent senescence [73] and JNK is required for expression of the JUN and JUND components of AP-1. Indeed, a reduction of Fos in several brain regions has been also correlated with physiological aging [74].

Considering this, we compared miR-335 expression levels in a panel of hMSCs obtained from donors ranging from 18 to 55 years old, and found a direct correla-

tion between miR-335 expression level and the age of the donor. Therefore it seems plausible that miR-335 levels could be a good marker of physiological aging. Consistent with this, previous work [75] demonstrated that miR-335 and miR-452 are the only two miRNAs that are upregulated in hMSCs obtained from adipose tissue of old donors in comparison with young donors. Additionally, this study demonstrated that aged hMSCs had reduced levels of FOS, JUN and ERK1/2, together with increased expression of NF-kB, MYC and IL4R. This is in good agreement with the majority of the phenotypes associated with forced expression of miR-335.

Conclusions

This study demonstrates that miR-335 must be downregulated to allow hMSCs to respond to regulatory and differentiation signals. Aging or prolonged culture of hMSCs induce a progressive and constitutive increase of miR-335 levels, rendering these cells less responsive to activation stimuli (pro-inflammatory and/or pro-differentiation signals), and more prone to aging/senescence. This state, which is reversed by telomerase, translates to an impaired capacity for cell migration, and is linked to reduced AP-1 activity. As miR-335 expression levels are closely linked to the therapeutic activity of hMSCs, we consider that this miRNA might be used as a valuable predictive marker to monitor therapeutic potency of individual preparations of hMSCs-based medicinal products.

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Disclosure Of Potential Conflicts Of Interest

MT, MAG, and AB are inventors of a patent application in part based on findings described in this article. The other authors have no potential conflicts of interest.

Author Contributions

M.T.: Collection and/or assembly of data, manuscript writing; J.C.S.: Collection and/or assembly of data; M.D.: Collection and/or assembly of data, data analysis and interpretation; J.A.A.: Collection and/or assembly of data, data analysis and interpretation; J.C.: Data analysis and interpretation; M.A.G.: Conception and design, financial support, data analysis and interpretation, manuscript writing; A.B.: Financial support, data analysis and interpretation, manuscript writing and final approval of manuscript.

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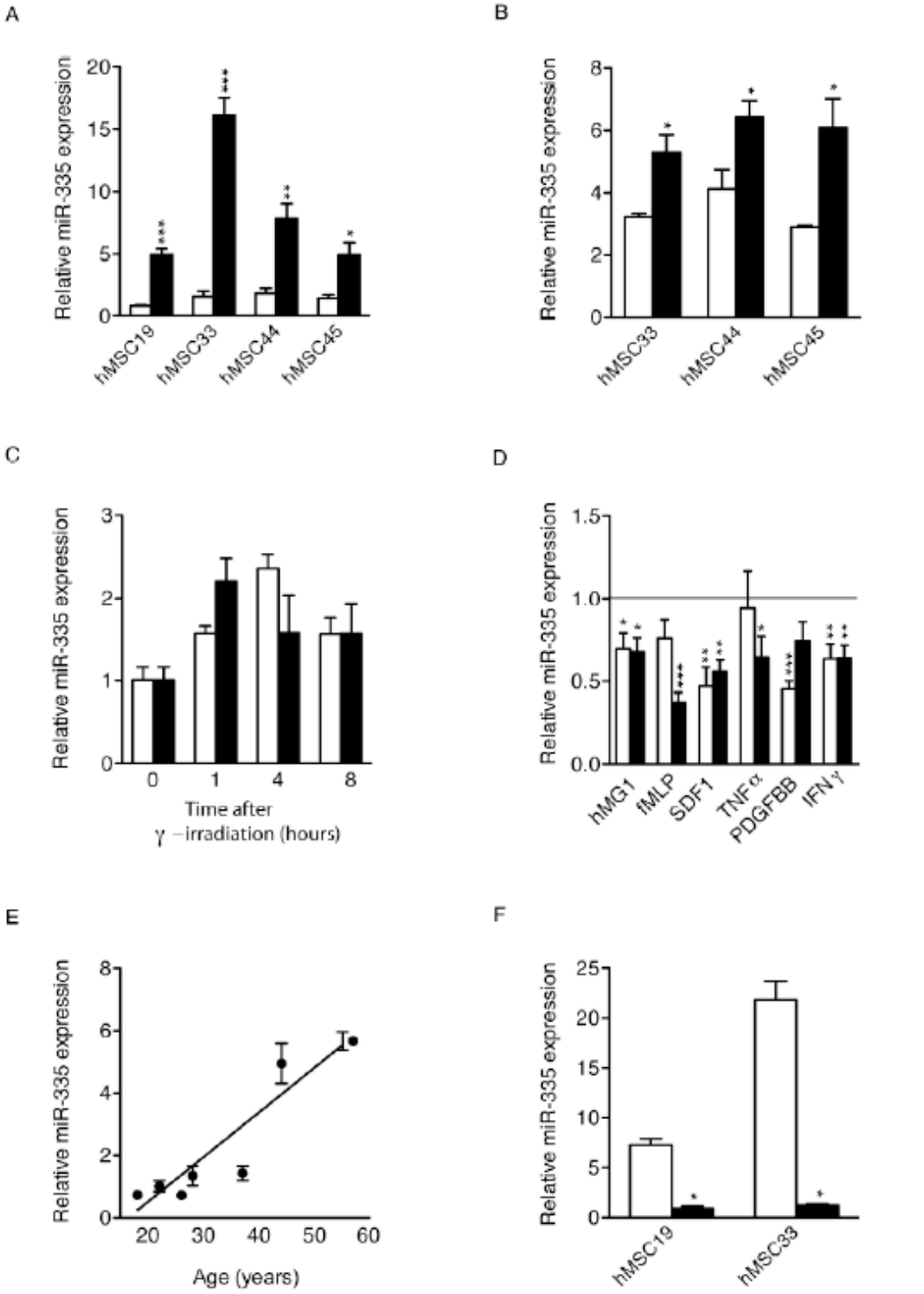


Figure 2. Forced expression of miR-335 induced senescence-related changes in hMSCs. Bone marrow-derived hMSCs were transduced with the lentiviral vectors pLV-EmGFP-MIR335 or pLV-EmGFP-mock (encoding a negative control shRNA), and transduced (gfp+) cells were purified by FACS. The purified cells (335- and control-hMSCS, respectively) were used to analyze different markers previously associated with senescence in hMSCs. **(A):** Percentage of proliferating 335- and control-hMSCs by EdU imaging. White bars, control-hMSCs; black bars, 335-hMSCs. **(B):** Alterations in cell size and morphology. Left panel, representative images showing alterations in morphology and cell size similar to those seen in senescent cells, scale bar represents 100 μm. Middle panel: cell size was measured by FSC-A using FACS. Right panel: DAPI-stained nuclei were analyzed for size and irregularity using the NMA tool, as described in materials and methods, and the percentage of small and regular nuclei (SR) is shown. At least 200 nuclei were analyzed for each data point. White bars, control-hMSCs; black bars, 335-hMSCs. **(C):** SA-β-gal staining of senescent cells induced by overexpression of miR-335. White bars, control-hMSCs; black bars, 335- hMSCs. **(D):** Lamin B1 expression was quantified in 335- and control-hMSCs by real-time RT-PCR (α-tubulin as endogenous control). White bars, control-hMSCs; black bars, 335-hMSCs. Representative immunofluorescence images of Lamin B1 expression in 335- and control-hMSCs are shown in the left panel; scale bar represents 100 μm. **(E):** Real-time RT-PCR quantification of mRNA transcripts in 335- versus control-hMSCs (GAPDH as endogenous control). **(F):** Western blot of p53, p21, p16, CCND1, SOD2 and β-actin. The lower histogram shows protein quantification by pixel density analysis using ImageJ software, normalized to the β-actin signal. **(G):** Immunofluorescence staining of p16 in 335- and control-hMSCs. DAPI staining was used to define total cell counts. The percentage of p16 positive cells is shown in the lower panel; scale bar represents 100μm; white bars, control-hMSCs; black bars, 335-hMSCs. *p<0.05; **p<0.005; ***p<0.001. Error bars represent s.e.m. (N=3).

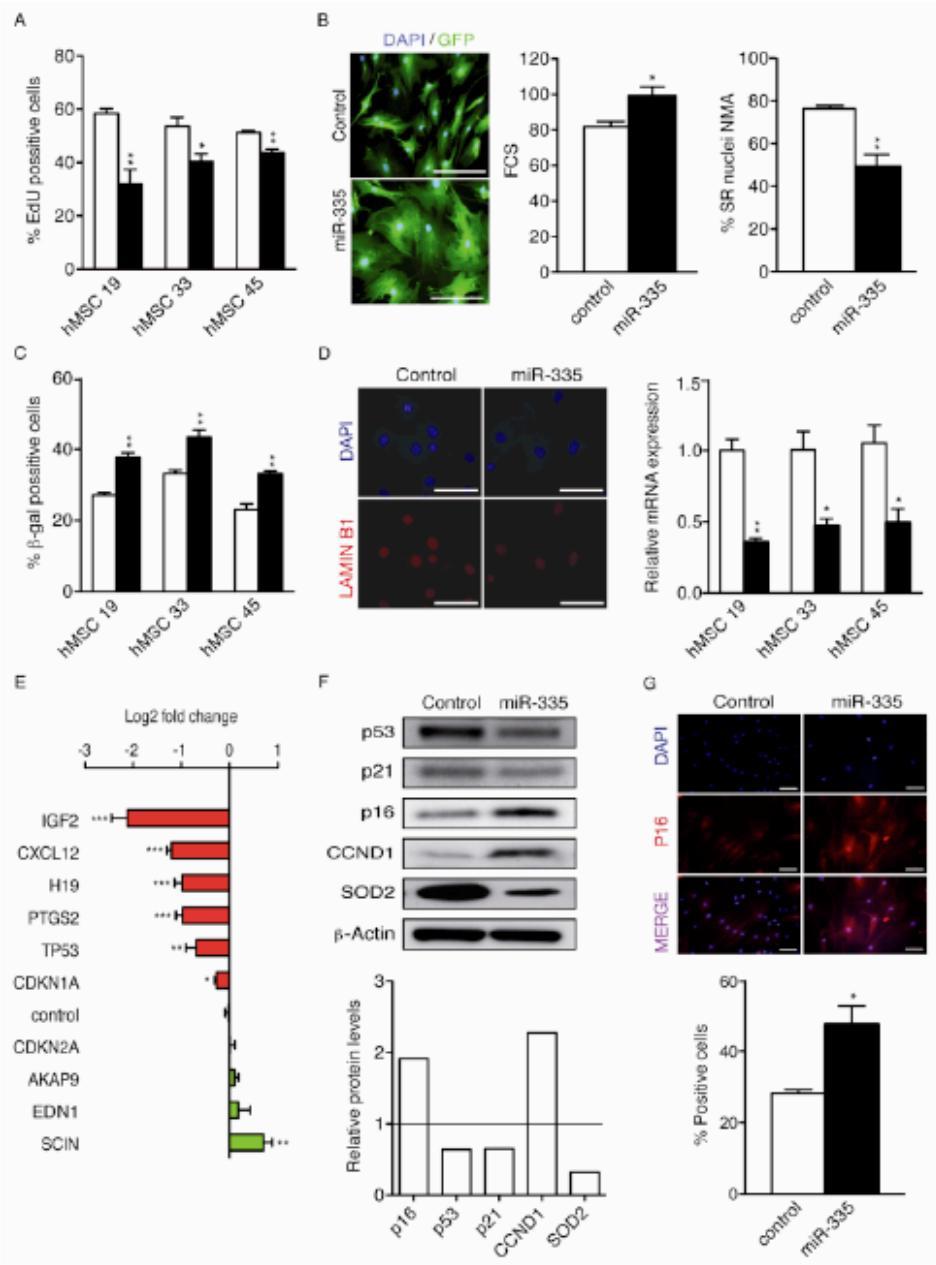


Figure 3. miR-335 promotes the acquisition of a senescence-associated secretory phenotype (SASP). **(A):** Conditioned medium (CM) from control- (white bars) and 335-hMSCs (black bars) obtained from three different donors was collected 24 h after serum starvation. Concentration of cytokines was analyzed by Luminex® multiplex assay. Protein levels were normalized to 10^5 cells/ml. Significant changes in protein levels are shown. **(B):** Relative IL6 and IL8 expression levels were quantified by real-time RT-PCR (α-Tubulin as endogenous control). **(C):** Significant changes in protein levels as measured by the same Luminex® multiplex assay in conditioned medium from control- (white bars) and 335-hMSCs (black bars), treated with LPS. *p<0.05. Error bars represent s.e.m. (N=3).

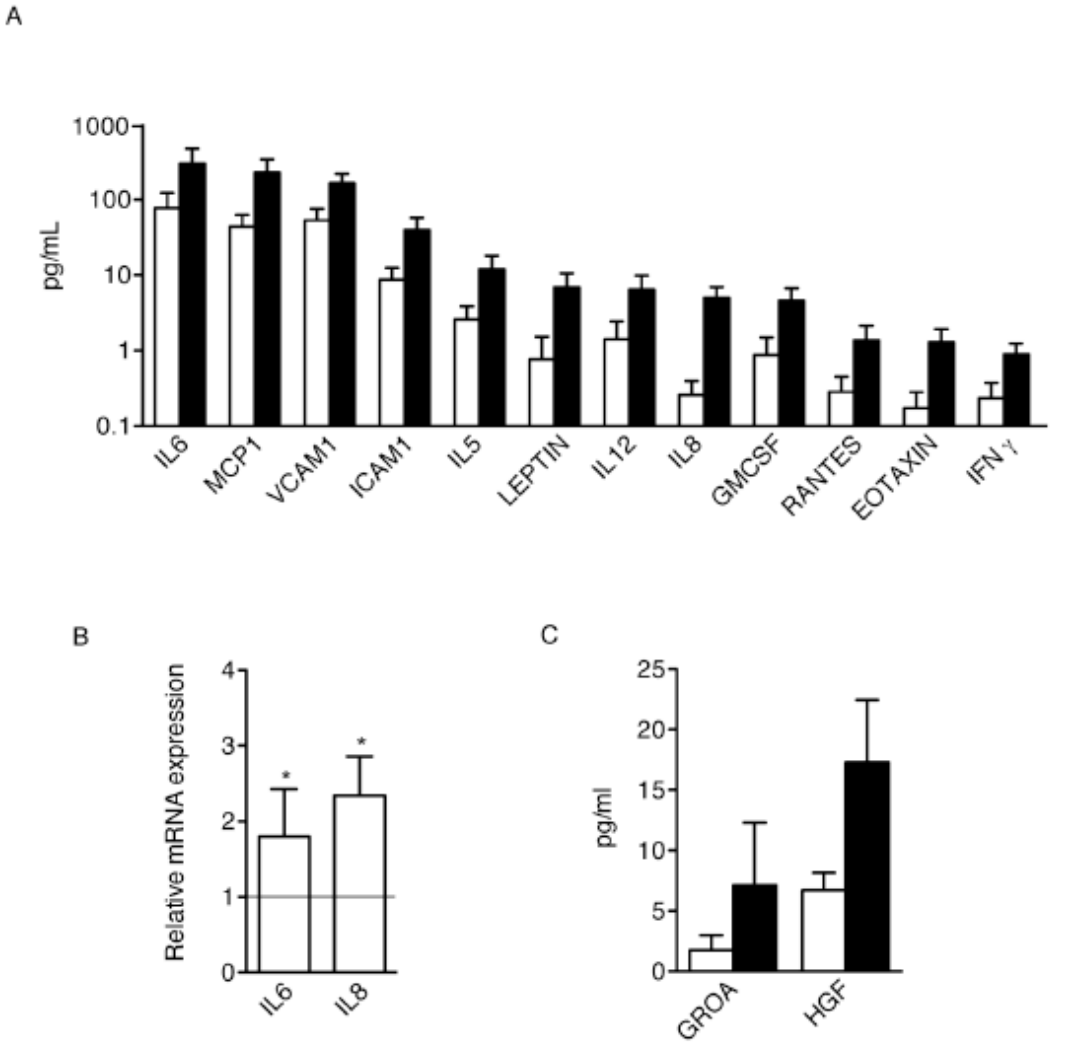


Figure 4. Forced miR-335 expression increases mitochondrial mass and ROS levels in hMSCs. **(A):** Upper panels, flow cytometry histograms of mitochondrial mass measured using MitoTracker®, and total and mitochondrial reactive oxygen species measured by DHE (di-hydroethidium) and MitoSOX™, respectively, in 335-(black histograms) and control-(white histograms) in hMSCs. Lower panels, relative fluorescence levels in 335-hMSCs shown as the percentage of control-hMSCs. **(B):** Relative fluorescence levels of mitochondrial reactive oxygen species measured with MitoSOX were normalized with fluorescence levels of mitochondrial mass using MitoTracker. Results in 335-hMSCs are shown as the percentage of control-hMSCs. **(C):** Representative confocal microscopy images of control- and 335 hMSCs after staining with MitoTracker, MitoSOX and DAPI. Color scale shows false color referring to increasing fluorescence MitoSOX:MitoTracker values, from lowest (blue) to highest (red), scale bar represents 500µm. *p<0.05; **p<0.005. Error bars represent s.e.m. (N=3).

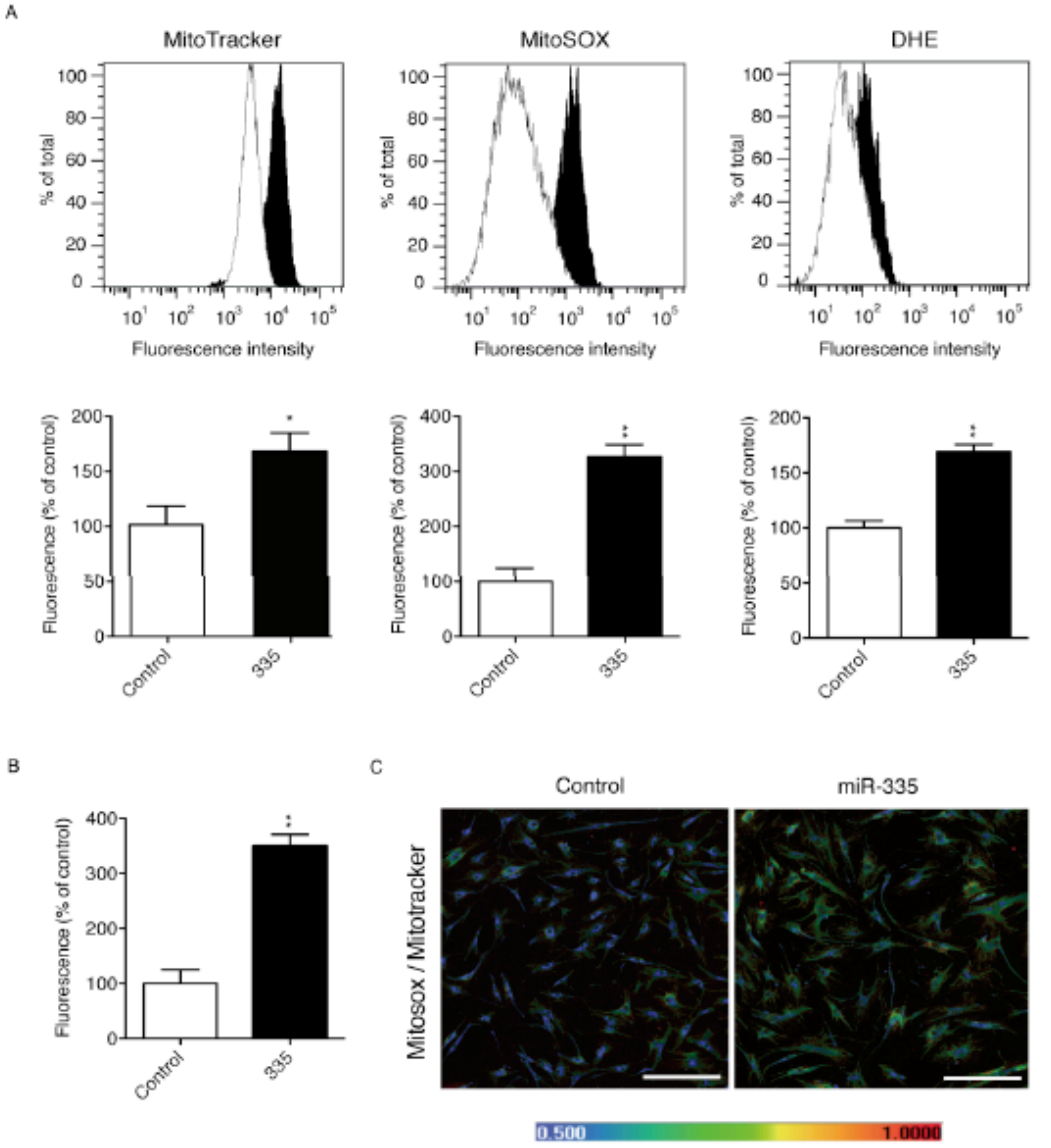


Figure 5. Chondro-osseous formation in hMSC-seeded diffusion chambers *in vivo*. *In vitro* predifferentiated preparations of control- **(A)** and 335-hMSC **(B)**, by stimulation with BMP2 in a collagen-gel medium containing 0.5% fetal bovine serum (FBS) for 10 days, followed by culture for 6 days in medium containing BMP2 and 10% FBS, were placed inside diffusion chambers and implanted subdermically into the back of 8-week-old rats for 28 days. Photomicrographs of histological sections of diffusion chambers, four weeks after implantation, using different stains are shown. Asterisks mark the wall of the chamber. Picrosirius-Hematoxylin was used for detection of collagen under polarized light (panels 1, 2, 3, 10, 11, 12); panels (2) and (11) show higher magnification images, marked by dotted boxes in (1) and (10), respectively; (4) matrix calcification evidenced by Von Kossa staining; (5) cartilage tissue stained with Alcian Blue; (6, 9) anti-Collagen Type I staining; (7) collagen fibers in the bone matrix detected using Picrosirius Red staining; (8) cartilage-like matrix stained with Toluidine Blue. Representative images from four different animals/treatment are shown. Scale bars: panels 1 and 10, 1 mm; 2 and 11, 500 µm; 3 and 12, 200 µm; 4-9, 100 µm.

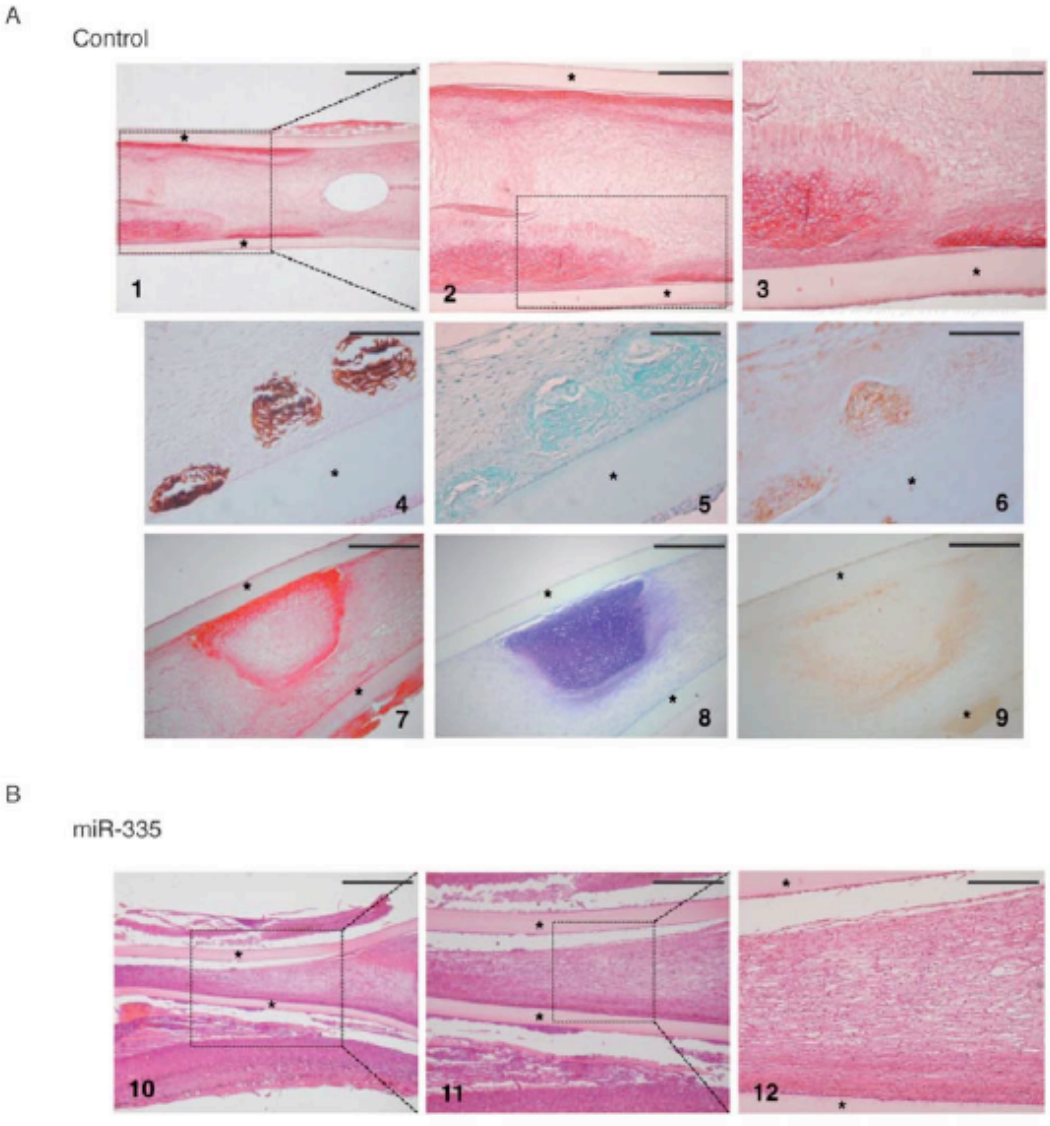


Figure 6. miR-335 overexpression inhibits the immuneregulatory effect of hMSCs. **(A):** Human PBMCs were stimulated or not (NS, non-stimulated) with phytohemagglutinin (PHA, 10 µg/ml), in the presence of different ratios of 335- or control-hMSCs (0:1 to 1:5 MSC/PBMC ratios). After 72 h of culture, proliferation was evaluated by BrdU incorporation. Gray bars, absence of hMSCs; white bars, control-hMSCs at the indicated ratios; black bars, 335-hMSCs at the indicated ratios. **(B):** Survival curves of mice (10 mice/group) after LPS injection and injection of 335- or control-hMSCs (PBS was used as a negative control). **(C):** Macrophages treated with LPS, were co-cultured with 335- or control-hMSCs. Macrophages cultured in the absence of LPS and hMSCs (NS) were used as negative controls. The co-culture was performed in the same well (cell-to-cell contact, upper panels) or in different compartments separated by a 0.8 µm-pore membrane using a transwell system (transwell, lower panels). Cytokine levels in the medium were determined after 24 h, by ELISA. **(D):** The migratory capacity of 335-hMSCs was quantified using a transwell migration assay. Different chemokines were used as stimuli. **(E):** CXCR4 expression levels measured by real-time RT-PCR in 335- and control-hMSCs (GAPDH as endogenous control). **(F):** PTGS2 expression levels measured by real-time RT-PCR in 335- and control-hMSCs after M ϕ-CM treatment at different time points (GAPDH as endogenous control). *$p<0.05$; **$p<0.005$, ***$p<0.001$. Error bars represent s.e.m. (N=3 in A, C-F).

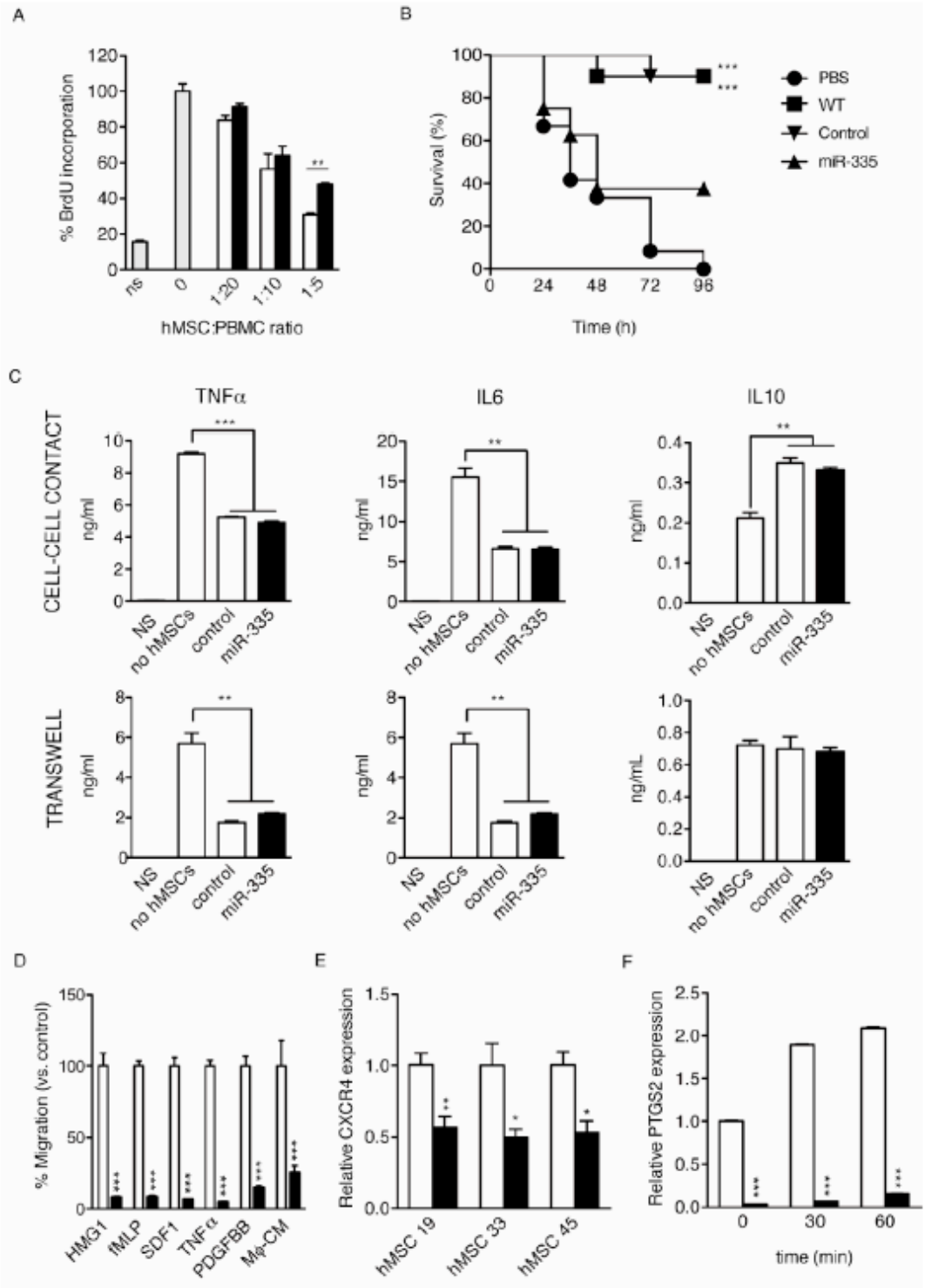
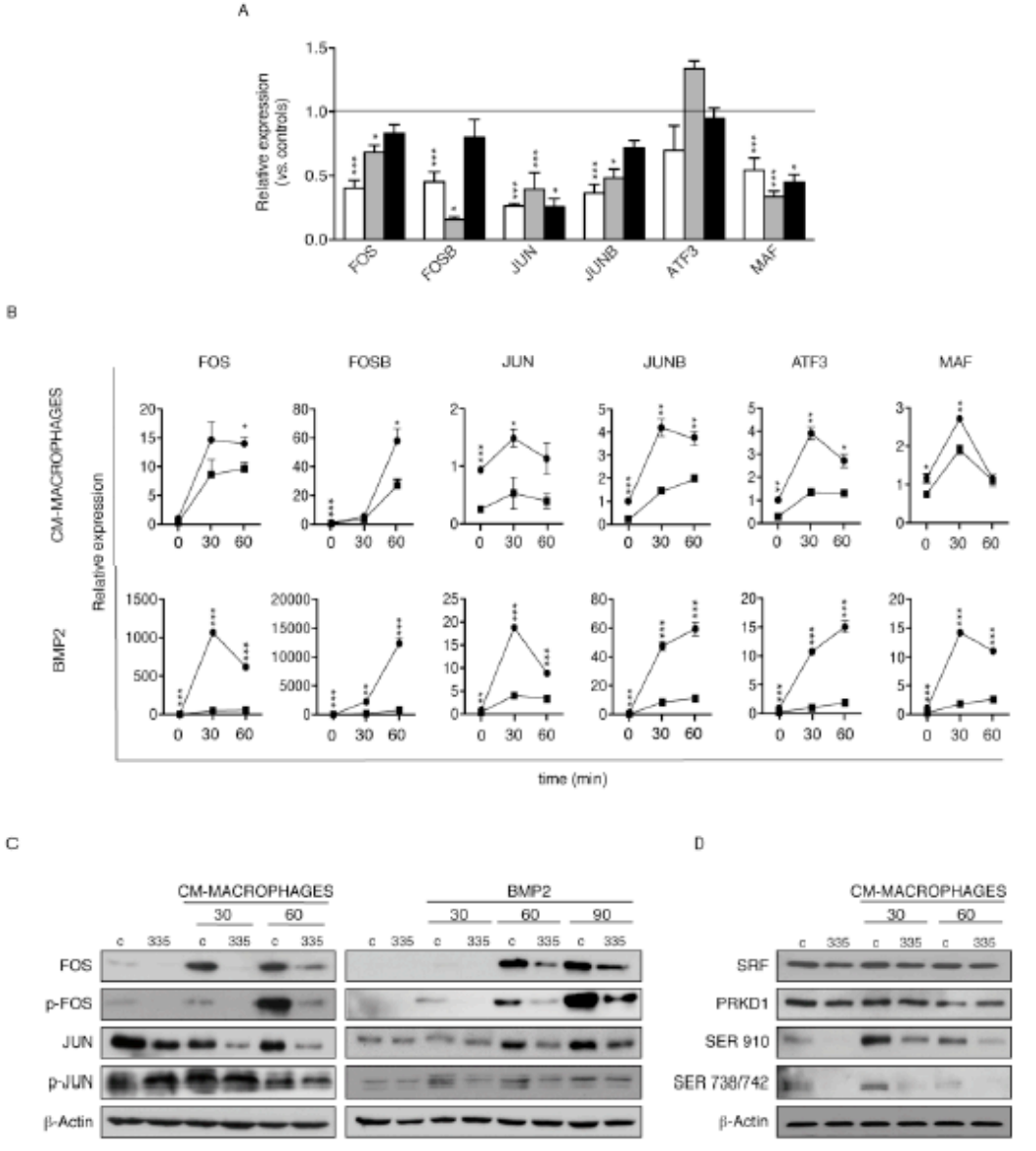


Figure 7. AP-1 activation in response to migration and differentiation stimuli is modulated by miR-335. **(A):** Relative basal expression levels of AP-1 components (MAF, ATF3, JUN, JUNB, FOS, FOSB; GAPDH as endogenous control) measured by real-time RT-PCR in 335-hMSCs from three different donors, versus the corresponding control-hMSCs. mRNA expression levels are presented as the relative fold-increase compared with control-hMSCs (horizontal line). The different colored bars correspond to three independent hMSC isolates. **(B):** Relative expression levels of AP-1 components in control- (black circles) and 335-hMSCs (black squares) after treatment with Mϕ-CM or BMP2 at indicated time points, measured by real-time RT-PCR. **(C):** Western blot analysis of AP-1 components after Mϕ-CM (left panel) and BMP2 (right panel) treatments at indicated times. Representative results from at least three experiments are shown. **(D):** Western blot analysis of SRF, PRKD1, and phospho-specific PRKD1 SER910 and SER738/742 protein levels after Mϕ-CM treatment at indicated times. Representative results from at least three experiments are shown. *p<0.05; **p<0.005; ***p<0.005. Error bars represent s.e.m. (N=3).



3. La senescencia celular suprime las propiedades terapéuticas de las células madre mesenquimales humanas en el modelo de endotoxemia letal (Sepúlveda *et al.*, Stem Cells. 2014 Feb 4. doi: 10.1002/stem.1654. [Epub ahead of print]).

En este trabajo corroboramos que los mecanismos utilizados por miR-335 para reprimir las capacidades terapéuticas de las hMSCs son los mismos que disminuyen la funcionalidad de las hMSCs senescentes.

Las hMSCs senescentes pierden su actividad antiinflamatoria *in vivo* en el modelo de endotoxemia, y al igual que las que sobreexpresan miR-335 de manera exógena, presentan una drástica inhibición de la capacidad migratoria en comparación con las hMSCs presenescentes. Dicha inhibición, de forma similar a lo descrito para niveles elevados de miR-335, es mediada por la fuerte represión de la activación de los componentes del complejo AP-1 en hMSCs senescentes.

Por último, describimos que las hMSCs senescentes, al igual que las que sobreexpresan miR-335 de manera exógena, muestran un SASP que les permite secretar al medio una amplia batería de moléculas proinflamatorias muy relacionadas con los procesos de inflamación crónica, incluidos los asociados con el envejecimiento fisiológico.

De ésta manera, confirmamos que los cambios fenotípicos y funcionales inducidos por miR-335 son similares a los observados en hMSCs que han alcanzado la senescencia. Tomados en su conjunto, nuestros resultados demuestran la relevancia de miR-335 en la biología de las hMSCs en general, y en particular en su funcionalidad terapéutica.

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TRANSLATIONAL AND CLINICAL RESEARCH

Cell Senescence Abrogates the Therapeutic Potential of Human Mesenchymal Stem Cells in the Lethal Endotoxemia Model

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Key Words. Mesenchymal stem cells • miRNA • Cellular therapy • Immunotherapy

ABSTRACT

Mesenchymal stem cells (MSCs) possess unique paracrine and immunosuppressive properties, which make them useful candidates for cellular therapy. Here, we address how cellular senescence influences the therapeutic potential of human MSCs (hMSCs). Senescence was induced in bone marrow-derived hMSC cultures with gamma irradiation. Control and senescent cells were tested for their immunoregulatory activity *in vitro* and *in vivo*, and an extensive molecular characterization of the phenotypic changes induced by senescence was performed. We also compared the gene expression profiles of senescent hMSCs with a collection of hMSCs used in an ongoing clinical study of Graft Versus Host Disease (GVHD). Our results show that senescence induces extensive phenotypic changes in hMSCs and abrogates their protective activity in a murine model of LPS-induced lethal endotoxemia. Although senescent hMSCs retain an ability to regulate the inflammatory response on macrophages *in vitro*, and, in part retain their capacity to significantly inhibit lymphocyte proliferation, they have a severely impaired migratory capacity in response to proinflammatory signals, which is associated with an inhibition of the AP-1 pathway. Additionally, expression analysis identified PLEC, C8orf48, TRPC4, and ZNF14, as differentially regulated genes in senescent hMSCs that were similarly regulated in those hMSCs which failed to produce a therapeutic effect in a GVHD trial. All the observed phenotypic alterations were confirmed in replicative-senescent hMSCs. In conclusion, this study highlights important changes in the immunomodulatory phenotype of senescent hMSCs and provides candidate gene signatures which may be useful to evaluate the therapeutic potential of hMSCs used in future clinical studies.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent precursors of non-hematopoietic mesodermal lineages that are capable of clonal differentiation into both mesenchymal and non-mesenchymal lineages. Although isolated predominantly from bone marrow and subcutaneous fat, MSCs can be found in virtually all mammalian connective tissues (reviewed in Bernardo *et al.* [1]). Human MSCs (hMSCs) can be isolated easily from small tissue biopsies, and expanded *ex vivo* for many cell passages without significant loss of differentiation potential. Together, these properties mark hMSCs as ideal candidates for cell and genetic therapy. Indeed, in the last decade, hMSCs have been used to treat a variety of traumatic and degenerative disorders, including bone fractures, cartilage lesions, and myocardial infarction.

In addition to their robust differentiation potential, MSCs have a remarkable capacity for inhibiting the immune response; an activity known generally as immunomodulation or immunoregulation (reviewed in Gebler *et al.* [2]). This activity bestows MSCs with a hypoimmunogenic phenotype, allowing them to evade, at least temporally, the alloreactive immune response, even in xenogeneic conditions. This immunoregulatory capacity of hMSCs includes the inhibition of host T cell proliferation and cytokine production, B cell proliferation, NK cell activation, and dendritic cell maturation. By exploiting these characteristics, hMSCs have been used successfully in different experimental models to prevent allogeneic transplant rejection, and to treat various experimental autoimmune/inflammatory disorders such as graft versus host disease (GVHD), experimental autoimmune encephalitis, collagen-induced arthritis, and septic shock. This immunoregulatory capacity is also being tested in clinical studies of GVHD, Crohn's disease, and other human inflammatory disorders.

While the immunoregulatory capacity of MSCs is recognized, the specific molecular mechanisms which govern this ability are poorly understood. Several cell-to-cell and soluble signals have been identified as potential drivers of immune suppression by MSCs, including prostaglandin E (PGE), indoleamine 2,3-dioxygenase (IDO), NO, and IL-10, but none of these can explain fully the diverse immune modulatory activities of MSCs observed *in vivo*. Moreover, the immuno-suppressive ability of MSCs is not constitutive, but requires proinflammatory signals such as IFNγ, which induce important phenotypic changes in MSCs [3, 4].

Increasing evidence suggests that the regenerative capabilities of transplanted MSCs in damaged tissues, such as infarcted myocardium, is linked more to their paracrine activity (including anti-inflammatory actions), than to their potential for differentiation into specific cell lineages (reviewed in Liang *et al.* [5]). Accordingly, understanding the physiological and pathological factors that affect the immune modulatory activity of hMSCs is not only relevant for the treatment of autoimmune/inflammatory disorders, but should also be considered a key step in the development of effective hMSC strategies for the treatment of degenerative pathologies.

Previously, we validated the use of xenogeneic murine models of autoimmune and inflammatory diseases to study the immunoregulatory activity of hMSCs *in vivo*. We found that systemic infusion of hMSCs in mice significantly reduced the incidence and severity of experimental arthritis [6], colitis [7, 8], and sepsis [8], to a degree similar to that of syngeneic murine MSCs. These therapeutic effects were mediated through down regulation of both Th1-driven autoimmune and inflammatory responses; as hMSCs migrate to secondary lymphoid organs, they reduce the production of inflammatory cytokines and chemokines, and induce *de novo* generation of CD4+CD25+FoxP3+ Treg with capacity to suppress self-reactive T effector responses [9].

Although hMSCs are available from different tissues, their quantity in the body is relatively low. As most cell therapy protocols use a minimum of 20-100 million hMSCs per treatment (autologous transplantation), hMSCs need to be expanded *in vitro* for at least 4 to 8 weeks prior to transplantation. The length of this period, and the overall quality of the hMSC product, depends both on the isolation and culture methods used, and also the clinical history, age, and the genetic makeup of the donor. Still longer expansion regimens would be required for more clinically useful off-the-shelf allogenic hMSC therapies.

Recently, it has been demonstrated that human cells (fibroblasts and normal and tumorigenic epithelial cells), induced to senesce through replicative exhaustion, DNA damage, or other stresses, mount a severe pro-inflammatory response through the activation of a senescence-associated secretory phenotype (SASP, reviewed in Tchkonia *et al.* [10]). This SASP is characterized by a 10-800 fold overexpression and secretion of various proinflammatory chemokines including IL-8, IL-6, GM-CSF, GROα, GROβ, GROγ, ICAM-1 and MCP-1-4. Additionally, it is becoming more accepted that all *ex vivo* cell expansion procedures favor the accumulation of aneuploid cells, which is intimately associated with the progression of senescence [11-13]. Other studies describe a direct correlation between telomerase activity (which blocks cell senescence) and stem cell function (reviewed in Tümpel & Rudolph [14]). Therefore, it is evident that a pre-expansion test, which can anticipate the behavior of the hMSCs, would be a valuable tool to help improve expansion yields and, also to guarantee biosafety and functional value of the clinical product.

Considering that immunosuppressive activity is induced upon exposure of MSCs to proinflammatory signals, we hypothesized that this response will be dependent on the functional state of the MSCs. We show here that cell senescence strongly impairs the immunoregulatory capacity of hMSC *in vivo*, and suggest that examination of cell senescence markers could

be a useful approach to evaluate the clinical potential of hMSCs.

Materials and Methods

Biological samples

The study was carried out in accordance with guidelines of the Instituto de Salud Carlos III (Madrid, Spain). For the induction of senescence and related experiments, four independent hMSC lines (listed as #19, #33, #44, and #45), isolated from bone marrow tissue of male or female donors aged 18–29 years, were acquired from Inbiobank Stem Cell Bank (San Sebastián, Spain). For some experiments we also used eight bone marrow-derived hMSC samples obtained from an ongoing clinical study of graft-versus-host disease (GVHD). A brief description of the clinical study is included in the Supplementary Information.

Cell Culture

hMSCs ($4x10^3$ cells/cm^2) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin 100 U/ml and streptomycin 1000 U/ml (all culture reagents were from Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a humidified 37ºC incubator at 5% CO_2, and were passaged once per week. Media was changed twice weekly.

Induction of cell senescence

Cell senescence was induced by exposing cultured hMSCs to ionizing radiation (10 Gy) produced by a high-voltage X-ray-generator tube (Mark I-68, J.L. Shepherd & Associates, San Fernando, CA, USA). Senescence was assessed ten days after irradiation by cytochemical staining for ß-galactosidase, as described in Supplementary Information. The obtained radiation-induced senescent cells were denoted SEN+. Alternatively, in some experiments cells were grown until reaching replicative exhaustion (replicative senescent cells).

TERT lentiviral transduction

Primary hMSCs at passage 5 were transduced as described [15] with a lentiviral vector encoding the human telomerase reverse transcriptase catalytic subunit (pRRL.hTERT) [16]. Cells were evaluated for long-term cell proliferation, telomerase expression and activity, and telomere length after ≥10 passages (described in Supplementary Information). Immortalized cells were denoted SEN-.

Induction of endotoxemia and sepsis

Endotoxemia was induced in 7- to 10-week-old BALB/c male mice (Harlan Laboratories, Gannat, France) by i.p. injection of 400 µg/mouse LPS (from *E. coli* serotype 055:B5; Sigma-Aldrich). At 30 min after LPS injection, mice received an i.p. injection of PBS or one million hMSCs in PBS. Survival after LPS injection was monitored every 12 h for 6 days.

Serum, liver, lungs and small intestines were collected 6 hours after LPS administration and used for protein extraction and cytokine measurement. All experiments were performed in accordance with institutional guidelines for the Care and Use of Laboratory Animals in Research, and approved by CNIC.

PBMC proliferation assay

Buffy coat preparations were obtained from whole blood of healthy volunteers following the guidelines of the Centro de Transfusión de la Comunidad de Madrid, Spain. Human PBMCs were isolated from the buffy coats by density sedimentation on Ficoll-Hypaque (Sigma-Aldrich) gradients (20 min, 2000 rpm, at room temperature). Cells recovered from the gradient interface were washed twice in RPMI complete medium (consisting of RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, penicillin 100 U/ml, and streptomycin 1000 U/ml) and immediately used for culture.

PBMCs (10^5) were cultured in duplicate with RPMI complete medium in the presence of phytohemagglutinin (PHA, 10 µg/ml, Sigma-Aldrich) with or without various quantities of hMSCs ($2x10^3$ to $5x10^4$) in flat-bottom 96-well plates. After 72 hours culture, proliferation was evaluated using a colorimetric method of BrdU incorporation (Roche Applied Science, Mannheim, Germany).

Macrophage culture

Peritoneal exudate mouse cells were elicited by i.p. injection with 2 ml of 3% sterile sodium thioglycolate (Sigma-Aldrich) in 8-week-old BALB/c male mice (Harlan Laboratories). Peritoneal cells were obtained 3 days later by peritoneal lavage with cold PBS, washed in cold RPMI medium and cultured in RPMI complete medium at a concentration of 10^6 cells/ml. After 2 h at 37ºC, non-adherent cells were removed by extensive washing. At least 95% of the adherent cells were macrophages as judged by morphological and phagocytic criteria, and by flow cytometry analysis (CD11b and F4/80). Macrophage monolayers ($1x10^6$ cells/well) were incubated with RPMI complete medium in the absence or presence of LPS (1 µg/ml), and hMSCs (1:5 hMSC:macrophage cell ratio). To determine the cell-contact dependence of the co-culture response, LPS-stimulated macrophages ($1x10^6$) were placed in the upper insert of a transwell system (0.8 µm pore, Corning Inc., Corning, NY, USA), and hMSCs ($2x10^5$) were placed in the lower well. Cell-free supernatants were collected after 24 h incubation, and cytokine levels were determined. For the generation of macrophage-CM, macrophages were cultured for 24 h at 80% confluence in RPMI complete medium containing LPS (1 µg/ml).

Cell migration assay

To determine the *in vitro* migratory potential of hMSCs in response to signaling by stimulated macrophages, hMSCs ($1.5x10^4$) were cultured in RPMI complete medium in a 24-well tissue culture insert with an 8 µm pore size membrane (Corning). Inserts were placed on top of wells containing macrophage-conditioned media (macrophage-CM). For the generation of macrophage-CM, macrophages were cultured for 24 h at 80% confluence in RPMI complete medium containing LPS (1 µg/ml). After 3 h, the filter membranes were washed with PBS, and non-migrated cells were removed from the upper side using a cotton swab. Finally, membranes were fixed with 4% PFA and mounted in mounting medium containing DAPI. Migration of hMSCs was determined by counting the number of DAPI-stained nuclei on the underside of the membrane under x 200 magnification, using CellProfiler™ image analysis software (http://www.cellprofiler.org).

Cytokine determination

For cytokine determination in mouse tissues, protein extracts were isolated by homogenization of tissue fragments (50 mg tissue/ml) in 50 mM Tris-HCl, pH 7.4, with 0.5 mM DTT, and 10 µg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl fluoride, pepstatin and leupeptin (Sigma-Aldrich). Samples were centrifuged at 30,000g for 20 min and stored at –80ºC. Cytokine levels in the serum, tissue protein extracts and culture supernatants were determined by specific sandwich ELISAs using BD OptEIA ELISA Sets (BD Biosciences, Mississauga, Canada).

Secretome analysis

Subconfluent cultures (10,000 cells/cm^2) were washed and incubated in serum-free DMEM for 24 h to generate conditioned medium (CM), which was collected and cells counted. CM was filtered (0.2 µm pore), frozen at -80ºC, and later analyzed using a custom human 51-plex Luminex assay (Affymetrix, Santa Clara, CA, USA), as described in Supplementary Information.

Microarray analysis.

Total RNA was isolated from cultured cells with the miRNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified with a NanoDrop-1000 spectrophotometer and quality was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Agilent Whole Human Genome 4x44K V2 Microarray Kit (G4845A, Agilent Technologies), and Agilent Human miRNA Microarray V3 (G4470C, Agilent Technologies) were used to measure gene and miRNA expression, respectively. A full description of the samples, experimental procedures, data processing and statistical analysis used for both types of microarrays is included in the Supplementary Information. All microarray results have been submitted to the Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo; accession number GSE48662.

Gene and protein expression analysis

Total RNA was isolated and quantified as described for the microarray analysis. Human transcripts were quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using the corresponding TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). GAPDH was used as endogenous normalization control.

Western blot and immunofluorescence analyses were performed as described in Supplementary Information.

Statistical and functional analysis

Statistical analysis of experimental data was performed with Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA). All values are expressed as mean ± standard error of mice/experiment. Unless otherwise stated, differences between groups were analyzed by double-tailed t-test. Survival curves were analyzed by the Mantel-Cox log-rank test. Results were considered statistically significant at $p<0.05$.

Gene (or gene product) functional analysis was generated as described in Supplementary Information.

RESULTS

Cell senescence inhibits the lymphocyte-inhibitory activity of hMSCs

Cell senescence was induced in human bone marrow-derived hMSCs by gamma-irradiation (10 Gy). Ten days after irradiation, 90% of cells displayed a senescent phenotype as, measured by ß-gal expression (Supplementary Fig. S1A). These cells were denoted 'SEN+'.

To inhibit (prevent) senescence, hMSCs were immortalized by lentiviral transduction of hTERT. Both expression of hTERT and telomerase activity were positive in the transduced cells after >10 passages in culture (Supplementary Fig. S1B, C). Compared with wild-type, non-transduced hMSCs, cumulative population doublings of the transduced cells showed an absence of proliferative arrest over 12 cell passages (Supplementary Fig. S1E), together with significant telomere elongation (Supplementary Fig. S1D). These telomerase-immortalized cells were denoted 'SEN-'.

To determine the extent to which cell senescence could impair the inhibitory effect of hMSCs on lymphocyte proliferation, we first performed an *in vitro* PBMC proliferation assay. Human allogeneic PBMCs were cultured with PHA for 3 days in the presence or absence of wild-type or senescent (gamma-irradiated) hMSCs (SEN+). We found that, although the basal inhibitory activity was variable in the four samples tested, the inhibition of lymphocyte proliferation by hMSCs was significantly reduced in all samples cultured with SEN+ cells, compared to non-irradiated cells (Fig. 1). Howev-

er, SEN+ hMSCs still retained a significant lymphocyte inhibitory capacity *in vitro* (between 25% and 53% at a PBMC:MSC ratio of 5:1).

Senescence abolishes the therapeutic immunoregulatory activity of hMSCs *in vivo*

We and others have previously described that infusion of hMSCs has significant therapeutic efficacy in experimental-induced sepsis, and protects against mortality caused by endotoxin [8, 17]. We thus investigated whether cell senescence influences the protective effect of hMSCs in a murine model of lethal endotoxemia induced by high-dose endotoxin. Using three independent bone marrow hMSC isolates, we tested the protective effect of the administration with: wild-type short passage (<6 culture passages) non-senescent (WT) cells, short passage gamma-irradiated SEN+ cells and long-term passage (>20 culture passages) telomerase-expressing hMSCs (SEN-). Interestingly, compared to WT cells, SEN+ hMSCs lost the capacity to rescue mice from LPS-induced endotoxemic death, while SEN- cells showed a similar protective effect to WT cells (Fig. 2A). As demonstrated previously, the protective effect of hMSCs to endotoxemic death is mediated by down regulating the exacerbated inflammatory response, which is characteristic of this condition [8, 17]. As anticipated, administration of WT hMSCs resulted in a significant reduction of the inflammatory mediators TNFα, and IL-6, in the serum and lungs of septic animals (Fig. 2B). Similar findings were observed with SEN- cells (Fig. 2B). Furthermore, IL-10 was significantly decreased in the serum, but not lung, of animals administered with WT or SEN- cells. In contrast, animals administered SEN+ hMSCs had similar levels of inflammatory mediators to those detected in untreated septic mice (Fig. 2B). No significant changes in cytokine levels were detected in the liver or intestine of the treated animals compared to untreated septic mice (data not shown).

Radiation-induced senescent hMSCs maintain their immunomodulation activity on stimulated macrophages, but have a reduced migratory capacity

We next asked whether SEN+ hMSCs were regulating the inflammatory response through acting directly on inflammatory cells, as has been reported previously for wild-type hMSCs [8]. Thus, we cocultured hMSCs with mouse macrophages activated with 1 μg/ml LPS, and measured inflammatory cytokine production. Consistent with our findings *in vivo*, coculture of macrophages with WT or SEN- hMSCs inhibited the production of TNFα and IL-6 by macrophages under LPS stimulation, and this was accompanied by a significant increase in secretion of IL-10 (Fig. 3A). Notably, these results were mirrored with SEN+ cocultures (Fig. 3A). However, when hMSCs were cocultured with macrophages on transwells (0.8 μm pore size), macrophage TNFα and IL-6 production were inhibited similarly to that observed with direct contact, but IL-10 levels were unchanged, underscoring the requirement for cell contact [17]. Given that a central component of the anti-inflammatory activity of hMSCs is their capacity for migration to sites of inflammation (reviewed in Spaeth *et al.* [18]), we then used transwell assays (8μm pore size) to assess the migration of hMSCs in the presence of LPS-stimulated mouse macrophages. Interestingly, compared with wild-type cells, SEN- hMSCs had a significantly reduced migratory capacity (approximately 85% reduction) in response to stimulated macrophages (Fig. 3B). Collectively, these results suggest that, although SEN+ hMSCs appear to maintain most of their intrinsic modulatory activity on macrophages, their capacity for performing this *in vivo* is probably limited due to their reduced migratory potential. Similar results (over 75% reduction in migratory capacity) were obtained using replicative senescent (rSEN) hMSCs (Fig. 3C; see Supplementary Fig. S7 for characterization of replicative senescent hMSCs).

A senescence-associated secretory phenotype in hMSCs

Previous work has described that irradiated fibroblasts undergo permanent senescence growth arrest, and develop a senescence-associated secretory phenotype (SASP) 4-7 days after irradiation [19]. To determine the secretory phenotype of SEN+ hMSCs, we induced senescence in four independent isolates of human bone marrow-derived hMSCs by exposure to a similar dose of ionizing radiation (10 Gy). Ten days following irradiation, wild-type (WT, non-irradiated) and senescent (SEN+, gamma-irradiated) cells were cultured in serum-free medium for 24 h and this conditioned medium (CM) was collected. The CM was analysed using Luminex antibody arrays designed to detect 51 secreted human proteins involved in intercellular signalling during inflammation (Supplementary Table S1). Signals were calculated and quantified as described in Supplementary Methods, and presented as the MFI log2-fold changes between SEN+ and WT samples for each analyte. Consistent with studies in other cell types [19], we found that, although the profiles were in part donor dependent, senescent hMSCs (SEN+) secreted higher levels of numerous proteins compared to WT cells (Fig. 4A). Of the 51 proteins interrogated by the arrays, 27 were significantly altered (t-test, $p<0.05$) in the CM of SEN+ cells and were oversecreted in comparison to CM from WT cells. These 27 identified SASP components ranged from (normalized to 10^5 cells/ml) a low concentration of 0.92 pg/ml for IL-17F, to the highest concentration of 716.87 pg/ml for IL-6, in the CM of SEN+ cells (Fig. 4B). Furthermore, nine of the proteins (LEPTIN, TGFA, IL8, EOTAXIN, IFNG, VCAM1, IFNB, IL4, and MCP1) were secreted greater than 10-fold more from SEN+ cells compared to WT cells (Fig. 4C). The immune system processes (GO terms) associated to all the identified SASP components are shown in Supplementary Table S2.

In order to verify whether the alterations found in radiation-induced senescent hMSCs were representative for replication-induced senescence, we analyzed the expression of three major components of the SASP (IL6, IL8, and MCP1) by real-time RT-PCR. Results demonstrated that all three genes were also overexpressed in replicative-senescent hMSCs in comparison with presenescent cells (Fig. 4D).

Genome-wide gene expression profile analysis of radiation-induced senescent hMSCs.

To complement the secretome profile of hMSCs, we also measured global gene expression of hMSCs in a parallel study. Microarray analysis (Agilent Whole Human Genome Microarray Kit) indicated that a total of 5975 protein-coding genes (of which 4102 corresponded to annotated genes) were significantly regulated (adjusted $p<0.05$) in hMSCs 10 days after irradiation, when compared to non-irradiated cells. Gene set enrichment analysis using the PANTHER program revealed significant enrichment (Bonferroni multiple test-corrected p-value<0.05) for a wide variety of biological processes, including cell communication and immune system process (Supplementary Fig. S2). A detailed analysis using the IPA® software showed a predominant enrichment (Benjamini-Hochberg multiple test-corrected p-value<0.05) for biological functions involved in gene expression, cell cycle, and cancer (Fig. 5A). Other significantly enriched functions included cell death, cellular growth and proliferation, and cellular movement (Supplementary Fig. S3). The IPA analysis also showed a significant enrichment of canonical pathways involved in cancer, cell cycle, and DNA repair (Fig. 5B). The regulated genes were mapped to a total of 25 networks (Supplementary Table S5). The top functions related to the altered networks were: cell assembly and morphology, cell cycle, and DNA replication, recombination, and repair.

To identify miRNAs potentially regulated in senescent hMSCs, we obtained differential miRNA expression profiles of SEN+ hMSCs *versus* non-irradiated cells (Agilent Human microRNA Microarray v2.0). A total of 31 miRNAs were significantly altered in the SEN+ cells (adjusted $p<0.05$, Supplementary Table S6). The most prominently regulated family of miRNAs was the hsa-miR-17 cluster; strikingly, all members of this family were down-regulated in SEN+ cells. Those miRNAs prominently upregulated included miR-629-3p, miR-572 and miR-135a-3p (Supplementary Table S6). Analysis of the miRNA microarray data with the IPA software showed a predominant enrichment (Benjamini-Hochberg multiple test-corrected p-value<0.05) for cellular functions involved in cellular development, cell growth and proliferation, cell cycle, cell death, and DNA replication, recombination, and repair (Supplementary Fig. S4A, see Supplementary Fig. S5 for a full list of enriched biological functions). We also used the RNA22 tool to generate a list of putative targets for all of the regulated miRNAs, and obtained a non-redundant list of 5148 genes (4713 annotated). Of these, 3543 were assigned to 17 known GO biological processes by the PANTHER program (Supplementary Fig. S4B). Many of these processes could play a relevant role during the development of hMSC senescence. Interestingly, the second-highest score network identified through the use of IPA in the gene expression microarray data share MYC as their nodal molecule (Fig. 5C), in a network including 13 of the regulated miRNAs (Fig. 5D). Taken together our results show an extensive alteration in pathways related with cell proliferation and DNA damage response in SEN+ hMSCs, which is consistent with injury through irradiation.

A strong inhibition of the AP-1 pathway is associated with the migratory defect in senescent hMSCs

Based on our unpublished results, we hypothesized that the clear migration defect observed in senescent hMSCs could be related to the inactivation of AP-1. AP-1 mediates the response to a variety of extracellular stimuli and plays a key role in the regulation of diverse processes such as differentiation, proliferation and migration (reviewed in Angel *et al.* [20]). When we measured the expression of the AP-1 components FOS and JUN (and their phosphorylated forms) after stimulation of presenescent and both radiation- and replicative-senescent hMSCs with macrophage-conditioned medium (previously used as a migratory stimuli), we found that senescent hMSCs showed a marked decrease in the expression of these proteins (Fig. 6A).

To ascertain whether this defect in AP-1 activation could be related to the migratory impairment observed in senescent hMSCs, we evaluated FOS expression in an *in vitro* wound-healing assay (see Supplementary Information). The majority of presenescent wound-edge cells showed a high FOS expression 90 minutes after the scraping of the cell monolayer, whereas both irradiation- and replication-induced senescent wound-edge hMSCs exhibited comparatively weak FOS levels after the same time (Fig. 6B), These results demonstrate a close association of AP-1 inhibition with the impaired response of senescent hMSCs to migratory stimuli.

Clinical efficacy of hMSCs correlates with the expression levels of senescence-regulated genes.

Reminiscent of the results for our secretome profiling, it is recognized in the field that there is significant donor-dependent variability in the biological properties of hMSCs, which may limit their clinical value in allogeneic applications. Given our findings so far, we thought it instructive to determine whether the differentially regulated gene functions in experimentally-induced cell senescence could correlate with the clinical efficacy of hMSCs. We therefore performed microarray analyses of gene and miRNA expression in eight different hMSCs isolates (C1 through C8) which are being used in an on-

going clinical study (EudraCT number: 2009-011164-11). In this study, allogeneic MSCs obtained from bone marrow samples of healthy donors are being used to treat patients diagnosed of refractory severe acute or extensive GVHD.

For statistical analysis, the eight donor hMSC samples were classified in two groups depending on their clinical efficacy (see Supplementary Information for a summary of the clinical study and the major variables analyzed). Group 1 samples (Gr1: C1, C2, C4, C5, and C7) were those that induced a therapeutic response, either complete (C5), or partial (C1, C2, C4, and C7) at the lowest dose used in the study. Group 2 samples (Gr2: C3, C6, and C8) were those that did not elicit an effect that could be classified as a complete or partial response (see Supplementary Table S7). As might be expected from cells isolated from healthy donors and expanded under highly reproducible GMP cell culture conditions, no significantly regulated (adjusted $p<0.05$) gene functions (neither in protein-coding genes nor miRNAs) were found when comparing Gr1 and Gr2. However, a hierarchical cluster analysis of gene expression data revealed that two of the Gr2 samples (C4 and C8) were more similar to the SEN+ samples than to the control (WT) samples (Supplementary Fig. S6). We then searched for genes differentially up- or down-regulated in both SEN+ and Gr2 samples compared with WT samples, but not differentially expressed in Gr1 compared with WT or SEN+ samples. As shown (Fig. 7A), PLEC (upregulated; logFC=1.303), and C8orf48, TRPC4, and ZNF14, (downregulated; log FC=-1.202, -3.005, and -1.220, respectively) were selectively regulated in both Gr2 and SEN+ samples compared with WT hMSCs. None of these genes were similarly regulated in Gr1 cells compared to SEN+ or WT cells, which precludes the possibility of these differences being dependent on the slightly different culture conditions used for the clinical (Gr1 and Gr2) and non-clinical samples (WT and SEN+).

When real-time RT-PCR was performed with the same RNA samples used for the microarray experiments, only TRPC4 gave a statistically significant differential expression (Fig. 7B). Similar results were obtained when the relative expression of PLEC, TRPC4, and ZNF14 was evaluated in replication-induced senescent hMSCs (Fig. 7C). These results indicate that TRCP4 deserves further study as a potential quantitative marker for pre-evaluating the clinical efficacy of individual hMSCs samples used in future allogeneic clinical applications.

Discussion

Human mesenchymal stem cells (hMSCs) display a potent *in vivo* immunoregulatory activity, and are used with increasing frequency for cell therapy in the setting of autoimmune/inflammatory responses. Therefore, understanding the physiological and pathological factors that affect the immunoregulatory activity of hMSCs is of critical importance to develop safe and effective strategies for the treatment of degenerative disorders with an inflammatory component. Previous studies have clearly established that organismal aging correlates with a certain level of functional decline in hMSCs (reviewed in Sethe *et al.* [21]), including the reduction of proliferation, differentiation [22], and angiogenic [23] potential. This process has been related to phenotype alterations observed in culture, such as: morphological changes [24], reduction of telomere length [24, 25], expression of cell senescence markers e.g. p53 and beta-galactosidase [26-28], reduction in a subset of signalling molecules e.g. TGF-β and BMP2/4 [29], increased levels of proinflammatory molecules such as IL-6 [30], differential expression of several miRNAs [31], increased genetic damage [32], and decreased protection mechanisms such as repair [33] and antioxidant enzyme activities [34], and heat shock proteins [31]. However, a clear understanding of this process and the specific mechanisms involved in age/senescence-related loss of therapeutic potential in hMSCs is still lacking.

Here, we studied the potential relationship between the cell senescence process and the immunoregulatory activity of hMSCs, and attempted to assess the relevance of cell senescence markers as quantitative indicators for the clinical efficacy of hMSCs. We focused our investigations on cell senescence because: 1) due to the prolonged expansion regimens that are needed in the clinic to obtain sufficient amounts of hMSCs for therapy, it is quite likely that a substantial proportion of cells may undergo senescence, and 2) it is recognized that senescent human cells display important changes in their transcriptional and secretory states that confer them with strong proinflammatory characteristics [19].

Cellular senescence is a normal biological process which functions to prevent the proliferation of damaged cells, thus avoiding the transmission of damage to daughter cells. It consists of an irreversible cell growth arrest initiated in response to a range of intrinsic and extrinsic factors such as aging and exposure to stress. Ionizing radiation is a genotoxic agent that is able to induce cell senescence in a reproducible and persistent manner [35], without triggering cell death mechanisms such as apoptosis [36]. Our data demonstrate that radiation-induced senescence abrogates the protective immunoregulatory effect of hMSCs in a murine model of sepsis. Previous studies have shown that the mechanisms involved in the strong *in vivo* anti-inflammatory effects of hMSCs include the reduction of inflammatory cytokines and chemokines, the impairment of Th1 cell expansion, the induction of immunomodulatory macrophages, and the induction of regulatory T cells [6-10, 17, 37]. In agreement with these findings, systemic administration of hMSCs elicited a significant reduction in the serum and lung concentrations of TNF-β and IL-6 in our murine model. In contrast to data from other experimental models, we also observed a significant decrease in serum levels of IL-10 [6-8, 17]. The absence of a protective phenotype in senescent hMSCs correlated with the failure of these cells to cause significant reductions in cytokine levels in animals compared to those admin-

istered with hMSCs. However, senescent hMSCs retained some capacity for lymphocyte inhibition *in vitro*, and were comparable in their capacity to modulate the activation of macrophages, both by paracrine action and by cell-to-cell contact. Nevertheless, the migratory response of senescent hMSCs to signals from activated macrophages was severely reduced. Collectively, these results strongly suggested that the loss of *in vivo* immunoregulatory activity in senescent hMSCs is probably due mainly to their reduced migratory potential, rather than a dysfunction in their signalling mechanisms. Thus, administration of a senescent population of hMSCs in an inflammatory context could be biologically comparable to the administration of a very low dose of WT hMSCs. Infused senescent cells may modulate to a degree, the function of lymphocytes and macrophages, but too few cells would be able to reach the sites of inflammation to achieve clinical benefit. This reduction in the ability of senescent MSCs and other cell types to migrate has been reported previously [38-41], and may be related to changes in the cytoskeleton [42]. Our results suggest that this senescence-related migration defect could have important implications for the clinical use of hMSCs, and could be mediated by the inactivation of the AP-1 signaling pathway.

Human cells induced to senesce through extended proliferation or by genotoxic stress secrete many factors associated with inflammation (reviewed in Tchkonia *et al.* [10]). This senescence-associated secretory phenotype (SASP) develops over several days, and is triggered by persistent DNA-damage signalling [43]. The SASP described here for hMSCs was complex, and showed significant variability between donors, making interpretation of its potential biological effects challenging. Nevertheless, three proteins (IL-8, VCAM-1, and MCP-1) were secreted more than 10-fold in senescent cells compared to non-irradiated cells, reaching levels greater than 100 pg/ml. IL-8 induces chemotaxis in neutrophils and other granulocytes [44], and is a potent promoter of angiogenesis [45]. VCAM-1 mediates the adhesion of leukocytes to vascular endothelium, and the level of its soluble form has been frequently related to systemic inflammation [46, 47]. Finally, MCP-1 (CCL2) is a chemoattractant for monocytes and basophils and has a prominent role in several inflammatory diseases, such as multiple sclerosis [48] and inflammatory bowel disease [49]. The increased levels of these three molecules alone, notwithstanding other secreted pro-inflammatory mediators, strongly suggest that *in vivo* administration of senescent hMSCs could exacerbate the inflammatory response at a systemic level and/or counteract the anti-inflammatory effect of these cells as measured *in vitro*.

Cellular senescence changes profoundly the transcriptional profile of hMSCs [50, 51]. Our microarray analysis detailed more than 5000 genes, including 31 miRNAs, differentially expressed (adjusted $p<0.05$) in senescent hMSCs compared to control cells. Cellular functions prominently altered in senescent cells included cell growth and proliferation, cell cycle, cell death, and cellular movement. Furthermore, profiling of miRNA expression revealed an upregulation of the miR-34 family, which has been previously reported to be regulated by p53 and to induce cell senescence [52, 53]. In contrast, the miR-17 cluster was downregulated in senescent cells, mirroring it´s expression in human aging [54]. In our study, the pattern of senescence-regulated miRNAs was different from that previously reported in similar studies [31, 51, 55]. Whether these differences are related to the different species used, or are dependent on culture conditions or methods used to induce senescence (replicative versus radiation) etc, remains to be determined.

Notably, a significantly downregulated gene in senescent hMSCs was the proto-oncogene MYC, which also appeared as the nodal molecule in two of the highest scoring genetic networks identified from the gene expression microarray. MYC is a transcription factor whose activation by diverse mitogenic signals such as WNT, SHH and EGF results in cell proliferation. Moreover, reduced MYC signalling triggers telomere-independent senescence by regulating BMI1 and CDKN2A [56]. Indeed, the relevance of MYC down-regulation in hMSC senescence has been highlighted in previous data [57]. Thus, our results support the idea that radiation-induced MYC down-regulation in hMSCs could be an important mechanism for limiting the proliferation of damaged cells.

The complex transcriptional response triggered by senescence in hMSCs prevented any meaningful analysis to identify specific markers that could be relevant for the therapeutic efficacy of these cells. Also, our microarray data from hMSC cells used in an allogeneic clinical study failed to identify a single regulated gene when comparing therapeutically effective hMSC (Gr1) cells to clinically ineffective cells (Gr2). However, we did find that PLEC, C8orf48, TRPC4 and ZNF14 were similarly regulated in the latter group and in senescent (SEN+) hMSCs, when compared with WT control cells. PLEC (plectin, up-regulated in both Gr2 and SEN+ compared to WT) is a cytoskeletal cross-linking protein whose inhibition has been reported to bypass cellular senescence in rat embryo fibroblasts [58]. It is also described to be a major early substrate for caspase-8, which is required for actin reorganization during apoptosis [59]. The down-regulation of TRPC4 (a non-selective calcium-permeable cation channel) is also biologically relevant in the context of senescence, since it is known that replicative senescence leads to the suppression of calcium-dependent membrane currents in human fibroblasts [60]. Reassuringly, real-time RT-PCR validated the differential expression of TRPC4 in both radiation- and replication-induced senescent hMSCs. Therefore, we tentatively suggest that these four genes should be considered in further studies as potential markers for identifying hMSC samples with a low therapeutic potential in allogeneic clinical applications.

Conclusion

This present study provides evidence that cell senescence abrogates the protective activity of hMSCs in an experimental model of lethal sepsis. Interestingly, this loss of efficacy was not associated with a defect of hMSC-related inflammatory responses *in vitro*, but was possibly the result of a reduction in their migratory capacity associated with an inhibition of the AP-1 pathway. Senescent hMSCs also adopted a senescence-associated-secretory phenotype, directed to promote systemic inflammation. Candidate gene pathways dysregulated in senescent hMSCs included MYC, miR-34 family, and miR-17 cluster. In particular, four genes (PLEC, C8orf48, TRPC4, and ZNF14) were similarly regulated in senescent hMSCs, and in hMSC cells that were therapeutically ineffective in an allogeneic GVHD clinical study. The potential of these gene expression variations to act as markers for hMSC pre-evaluation will be addressed in future studies to gauge the therapeutic potential of hMSCs intended for use in clinical allogeneic applications.

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Author Contributions

Juan Carlos Sepúlveda: Collection and/or assembly of data, manuscript writing.; María Tomé: Collection and/or assembly of data.; María Eugenia Fernández: Provision of study material or patients.; Mario Delgado: Data analysis and interpretation.; Judith Campisi: Data analysis and interpretation.; Antonio Bernad: Financial support, editing and final approval of manuscript.; Manuel A. González: Conception and design, financial support, data analysis and interpretation, manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Figure 1. Senescent hMSCs have a reduced capacity to inhibit lymphocyte proliferation. Human PBMCs (10^5) were cultured in triplicate with complete RPMI medium in the presence of phytohemagglutinin (PHA, 10 µg/ml), and with or without various numbers of bone marrow-derived hMSCs (0:1 to 1:5 MSC/PBMC ratios) from different donors (hMSC19, 33, 44, 45) in flat-bottom 96-well plates. After 72 hours of culture, proliferation was evaluated by colorimetric measurement of BrdU incorporation. White bars, PBMC without hMSC; grey bars, PBMC cocultured with wild-type hMSC; black bars, PBMC cocultured with senescent hMSC; NS, non-stimulated PBMC. All data correspond to experiments performed using PBMCs from the same donor. *p<0.05; **p<0.01; ***p<0.005, versus cocultures with non-senescent hMSCs. Error bars represent s.e.m.

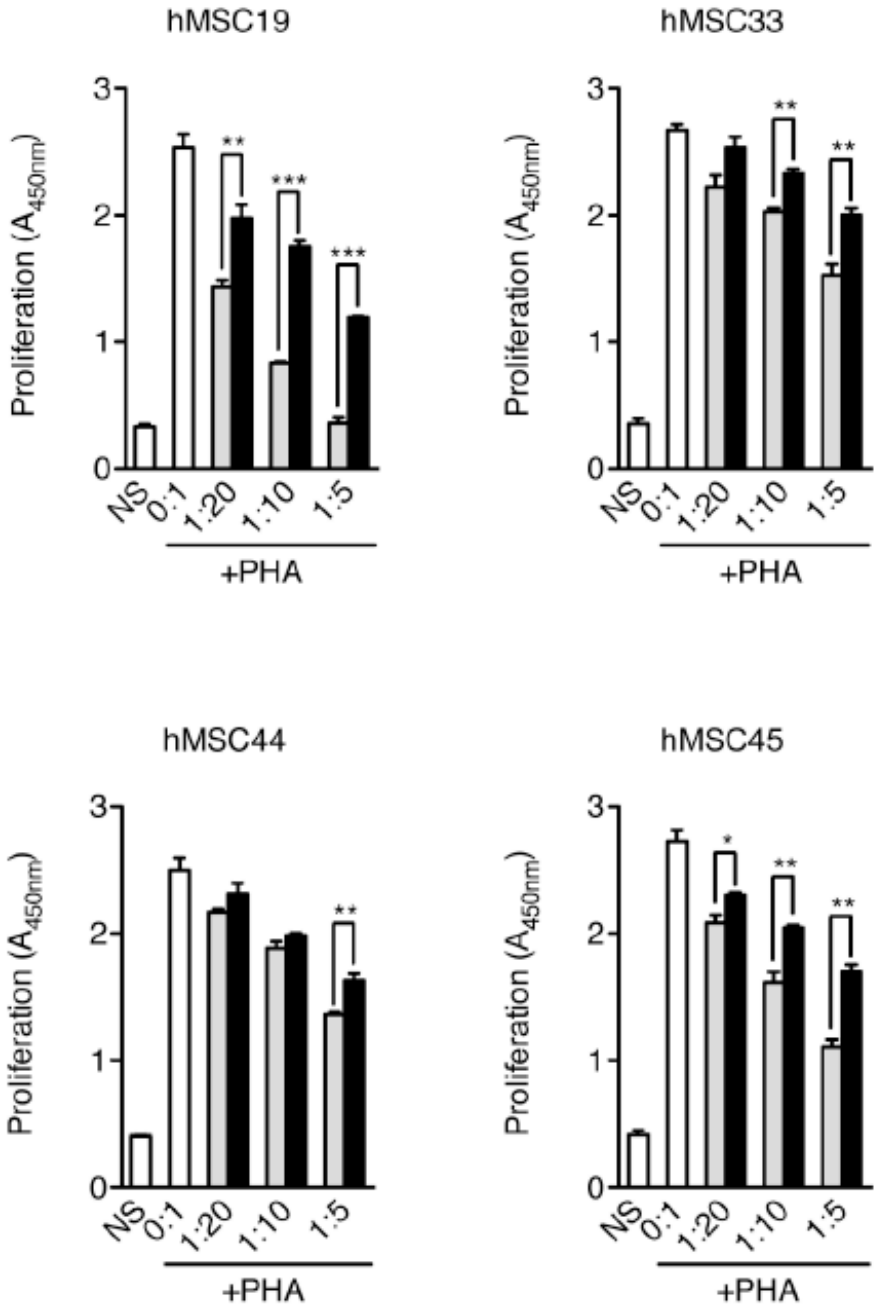
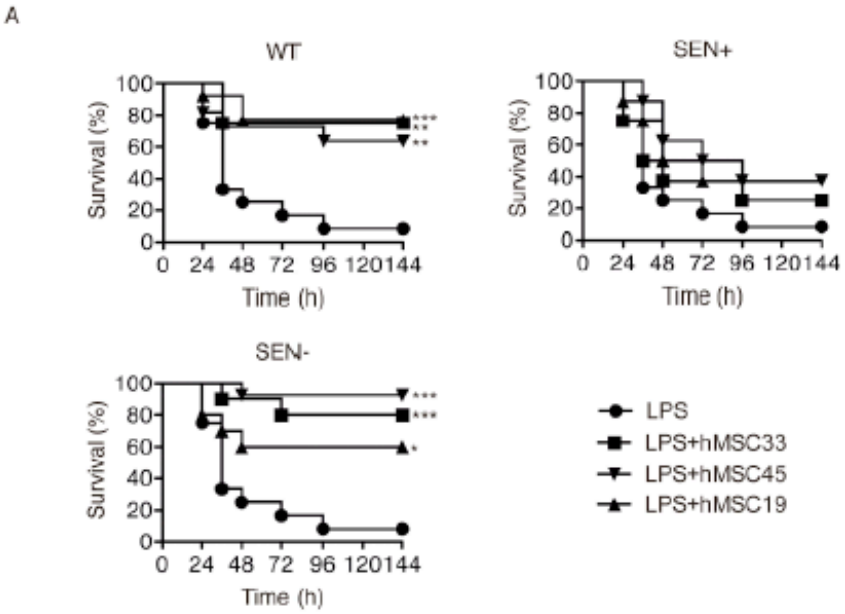


Figure 2. Senescent hMSCs fail to protect against lethal sepsis. Mice (10 mice/group) were injected i.p. with LPS (400 µg/mouse) and treated i.p. with PBS or 10^6 hMSCs 30 min later. Three different hMSC isolates (hMSC19, 33, and 44) were used. WT, wild-type primary hMSCs; SEN+, gamma-irradiated hMSCs; SEN-, telomerase-immortalized hMSCs. **(A):** Survival was monitored every 12h. **(B):** Cytokine levels were determined by ELISA in protein extracts from blood serum and lung collected 6h after LPS injection (N=5). *p<0.05; **p<0.01; ***p<0.005, versus controls with LPS alone. Error bars represent s.e.m.



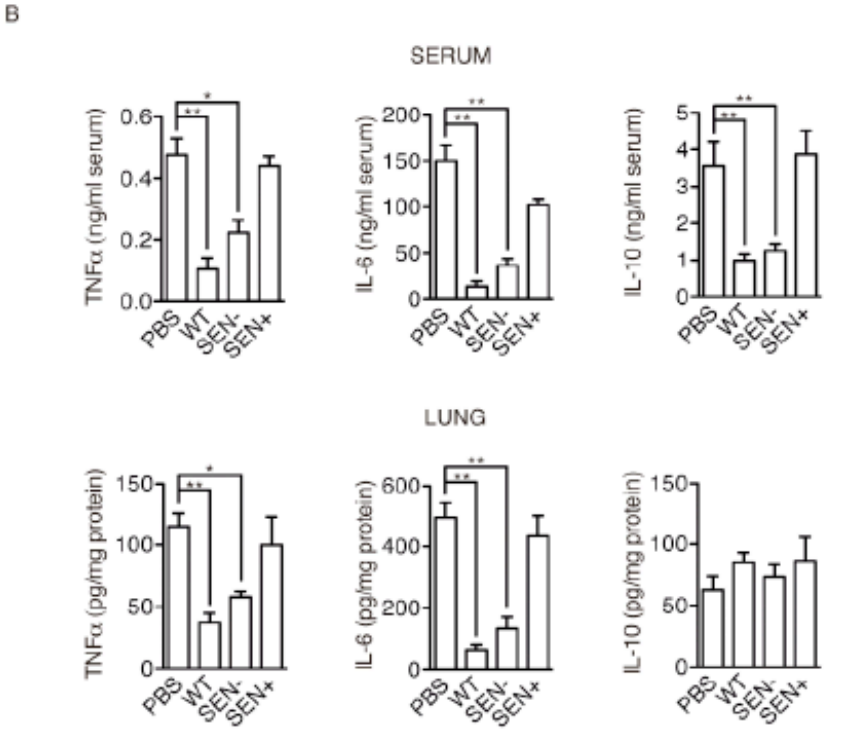


Figure 3. Senescent hMSCs maintain their intrinsic immunomodulatory activity on stimulated macrophages, but fail to migrate in response of proinflammatory signals. **(A):** Macrophages ($1x10^6$ cells/well) were cultured in the presence of LPS (1 µg/ml), alone (NC), or with hMSCs ($2x10^5$ cells/well). Macrophages cultured in the absence of LPS and hMSCs (NS) were used as negative controls. The experiment was performed with macrophages and hMSCs in the same well (upper panels) or with the macrophages and hMSCs separated by a 0.8 µm-pore transwell (lower panels). Cytokine levels in the medium were determined after 24 h, by ELISA. WT, wild-type primary hMSCs; SEN+, gamma-irradiated hMSCs; SEN-, telomerase-immortalized hMSCs. **(B):** $1.5x10^4$ presenescent (WT) or gamma-irradiated (SEN+) hMSCs were used in transwell migration assays in response to conditioned medium from LPS-stimulated macrophages. **(C):** $1.5x10^4$ presenescent (preSEN) or replicative senescent (rSEN) hMSCs were used in transwell migration assays in response to conditioned medium from LPS-stimulated macrophages. **p<0.01; ***p<0.005, versus controls. Error bars represent s.e.m. (N=3).

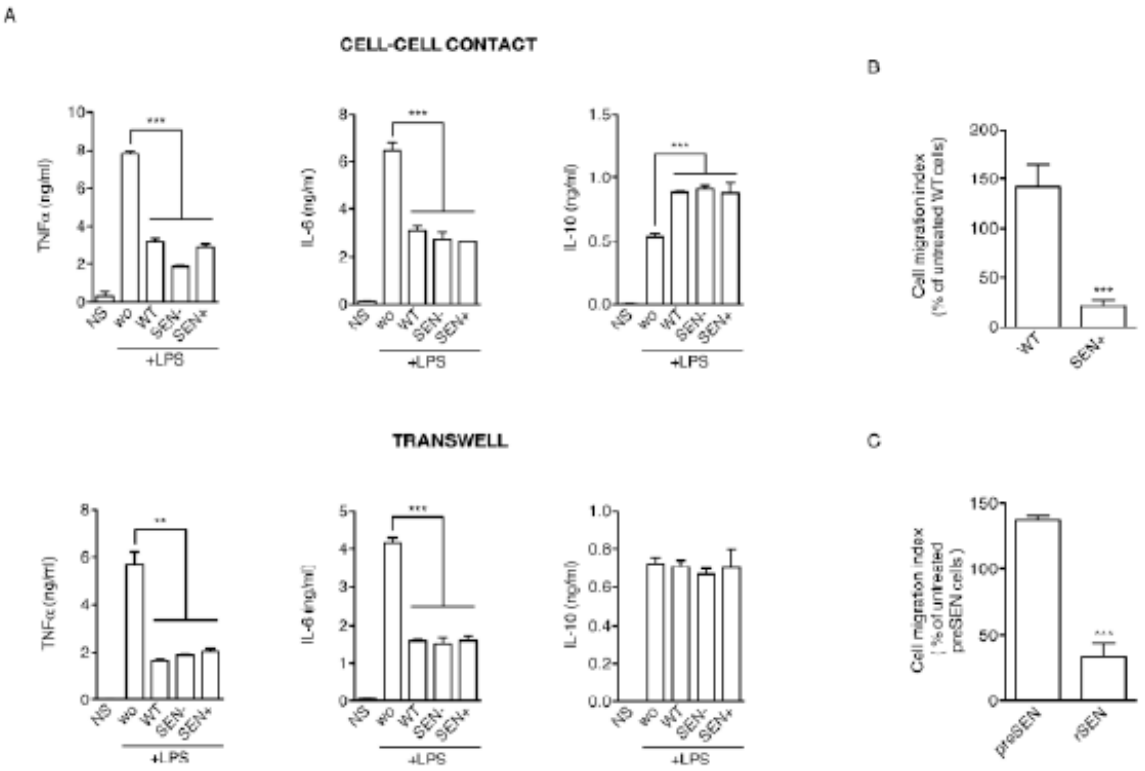


Figure 4. Senescent hMSCs secrete higher levels of inflammatory mediators compared to WT cells. **(A):** Soluble factors secreted by the indicated cells were detected by antibody arrays and analyzed as described in the text, Materials and Methods; and Supplementary Information. WT, wild-type primary hMSCs; SEN+, gamma-irradiated hMSCs. For each protein, all signals were averaged and used as the baseline. Signals higher than baseline are displayed in yellow; signals below baseline are displayed in blue. The numbers in the heat map key (right) indicates log2-fold changes from the baseline. Only proteins significantly oversecreted in SEN+ cells ($p<0.05$; compared with WT cells) are represented. **(B):** Levels of proteins (normalized to 10^5 cells/ml) significantly oversecreted in SEN+ cells compared with WT cells. **(C):** Fold changes in the levels of proteins significantly oversecreted in SEN+ cells compared with WT cells. When protein levels where under the detection limit, a baseline of 0.1 pg/ml was considered. **(D):** Relative expression levels of the major SASP components IL6, IL8, and MCP1 were quantified by real-time RT-PCR in three different isolates of presenescent and replicative senescent hMSCs. α-Tubulin was used as endogenous control. *$p<0.05$; **$p<0.01$; ***$p<0.005$, versus controls. Error bars represent s.e.m. (N=3).

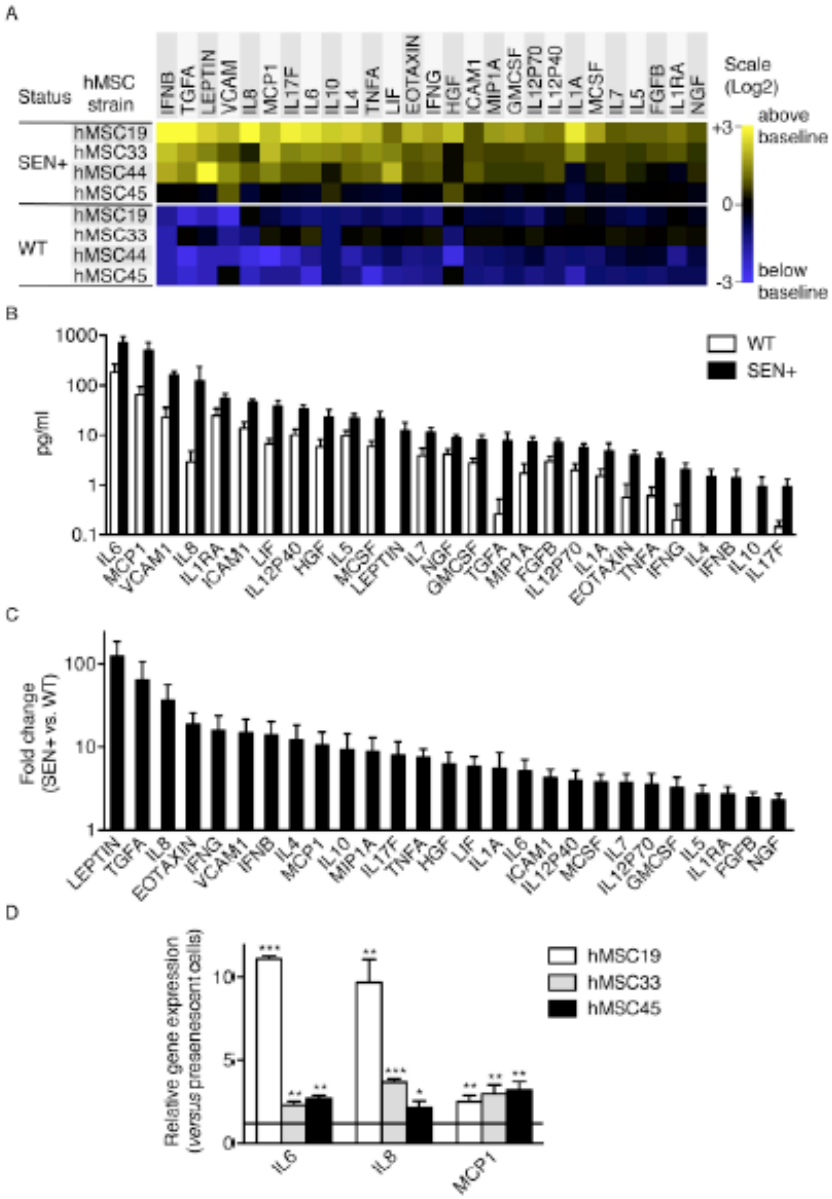


Figure 5. Gene function alterations found in SEN+ hMSCs. **(A):** Genes significantly regulated in SEN+ hMSCs (multiple-test adjusted p-value<0.05) in comparison with non-irradiated (WT) cells were analyzed using IPA (Ingenuity® Systems, www.ingenuity.com). Graphs show the top five functional categories altered in each of the three IPA major BioFunction groups. The full list of biological functions significantly regulated is shown in Supplementary Fig. S3. **(B):** Significantly regulated IPA canonical pathways (Benjamini-Hochberg multiple test-corrected p-value<0.05). **(C):** Top network generated using IPA from the list of differentially expressed miRNAs (SEN+ *vs.* WT hMSCs). Major functions associated with this network are: Cancer, reproductive system disease, and connective tissue disorders. Regulated genes appear shaded in green (down-regulated) or red (up-regulated). **(D):** Second most significant network found in the list of differentially expressed genes (SEN+ *vs.* WT hMSCs). Major functions associated with this network are: Cellular assembly and organization, cell cycle, and DNA replication, recombination, and repair (see Supplementary Table S5).

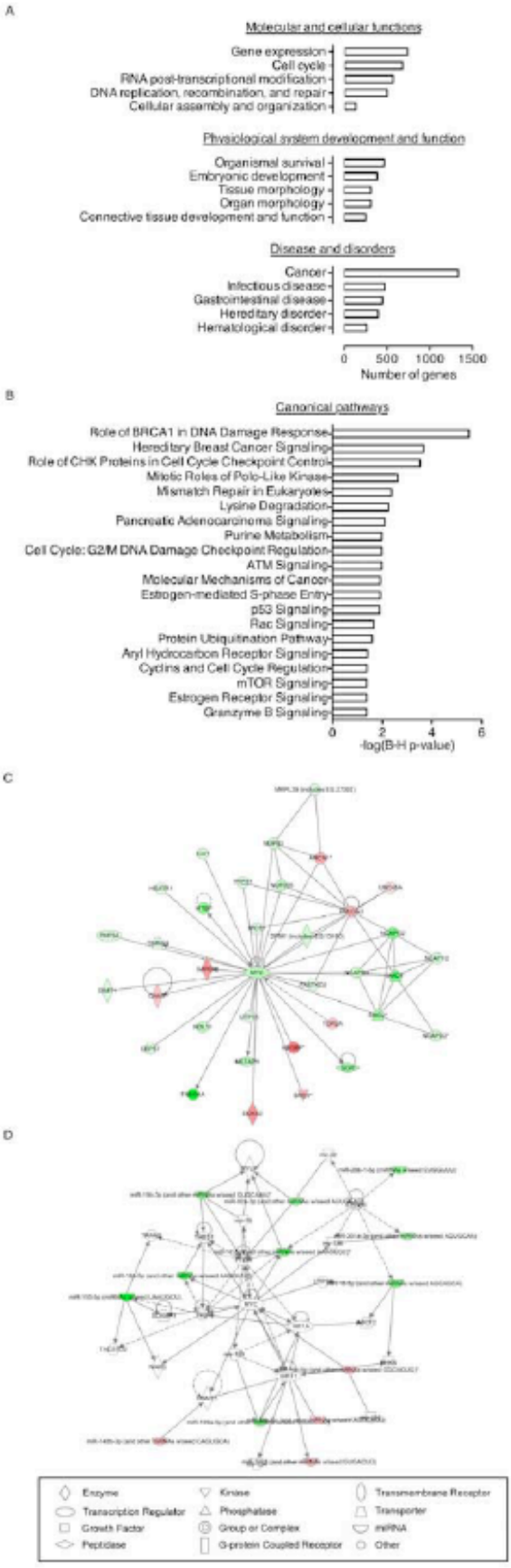


Figure 6. Senescent hMSC show a strong inhibition of the AP-1 pathway in response to migratory stimuli. **(A):** Western blot analysis of AP-1 components FOS, JUN, and their phosphorylated forms after treatment of presenescent (pre) or senescent (SEN) hMSCs with conditioned medium from LPS-stimulated macrophages at indicated times (minutes). Representative results from at least three experiments are shown. **(B):** Analysis of FOS activation at the wound edge. A scratch in a monolayer of presenescent (preSEN), radiation induced senescent (SEN+) and replication induced senescent (rSEN) hMSCs was made as described in Supplementary Information. At the indicated times after scratching (30 or 90 minutes), the cells were fixed, permeabilized, and stained with an anti-FOS antibody and the corresponding Cy5-labeled secondary antibody (red). Cell nuclei were counterstained using DAPI (blue). Representative fluorescence microscopy images with merged DAPI and Cy5 signals are shown. White dotted lines indicate the cell migration front (wound edge). White arrows point to some cell nuclei expressing FOS. Scale bar, 100 μm.

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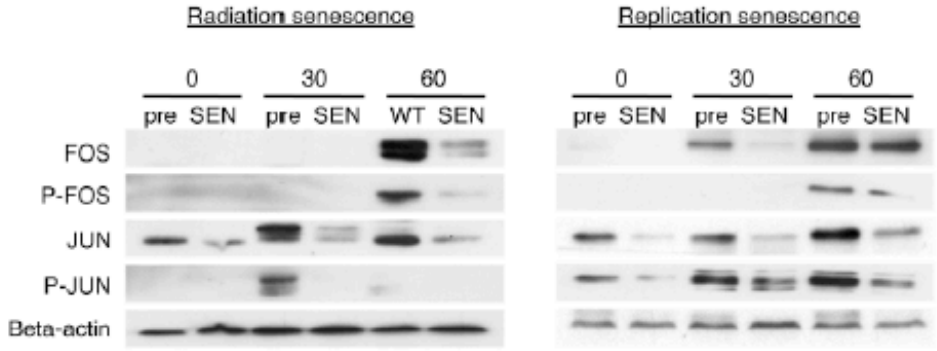

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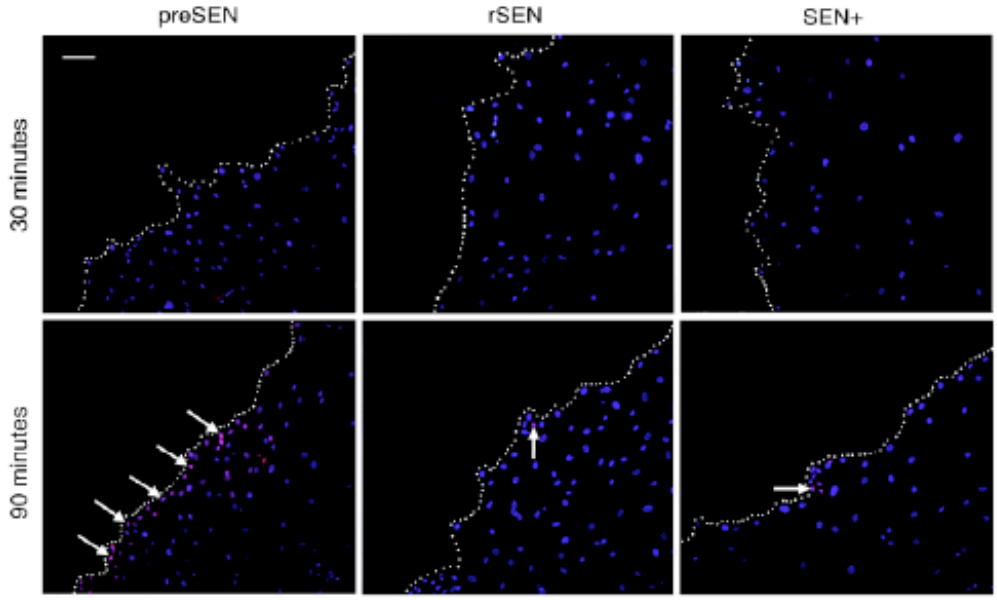

Figure 7. Four genes in the microarray analyses appear selectively regulated in both senescent and therapeutically ineffective hMSCs. We searched for genes differentially up- or down-regulated in both SEN+ and Gr2 samples (therapeutically ineffective) compared with WT samples, but not differentially expressed in Gr1 (therapeutically effective) compared with WT or SEN+ samples. **(A):** Venn diagrams show the number of RNA probes differentially expressed (adjusted $p<0.05$) in each cell population, analyzed with the Agilent Whole Human Genome Microarray Kit. For SEN+ and WT, N=4; for Gr1, N=5; for Gr2, N=3. **(B):** Relative expression levels of PLEC, TRPC4, and ZNF14 (endogenous control GAPDH) measured by real-time RT-PCR. The samples used were the same as those used for the microarray analysis. WT (average) was used as a calibrator for SEN samples, and Gr1 (average) was used as a calibrator for Gr2 samples. **(C):** Relative expression levels of PLEC, TRPC4, and ZNF14 (endogenous control GAPDH) measured by real-time RT-PCR in three different isolates of replicative senescent hMSCs. $^{**}p<0.01$; $^{***}p<0.005$, versus presenescent samples. Error bars represent s.e.m. (N=3).

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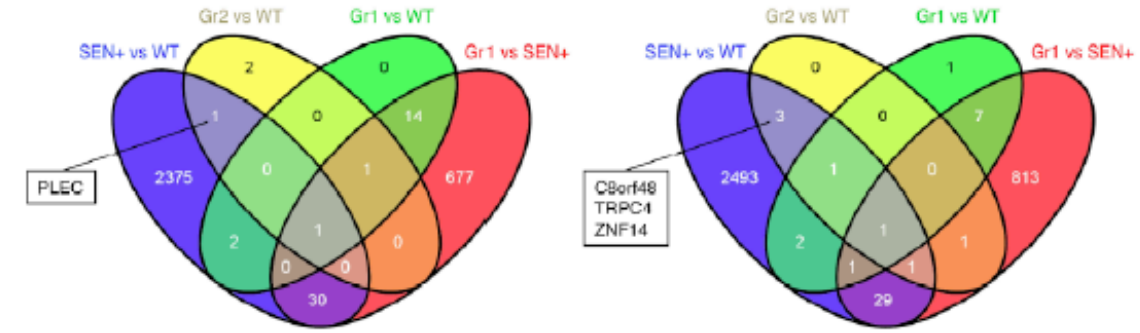

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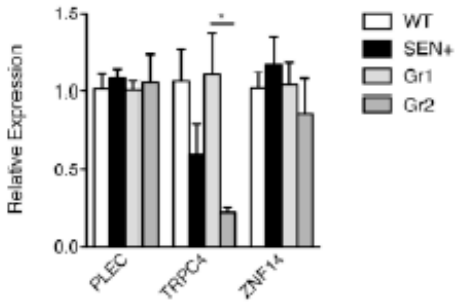

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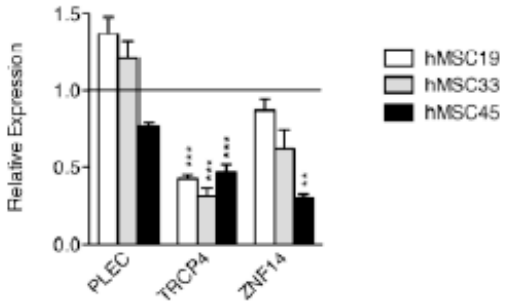

DISCUSIÓN

1. La disminución de la expresión de miR-335 es un mecanismo esencial para la activación de las hMSCs.

Para la identificación de miR-335 como principal regulador de la activación de las hMSCs se siguió un razonamiento similar al que había sido considerado anteriormente en el modelo de las células madre embrionarias[211]. Las hMSCs son células madre adultas que permanecen en un estado inactivo durante largos periodos de tiempo. Sin embargo, tienen la capacidad de responder a cambios ambientales y activar una amplia variedad de acciones reparativas[212, 213]. Los mecanismos de activación de las hMSCs ante diferentes estímulos parecen ser controlados por variaciones en las poblaciones de miRNAs, los cuales son capaces de regular la expresión de cientos de genes de forma simultánea[214, 215, 216]. Según esta hipótesis, en el estado inactivo de las hMSCs, uno o varios miRNAs inhibirían la expresión de un elevado número de genes, y su rápida regulación a la baja en respuesta a diversos estímulos permitiría la expresión de dichos genes, resultando en la iniciación de los diversos procesos celulares implicados en el mantenimiento de la homeostasis tisular, tales como proliferación, migración, diferenciación y regulación de la respuesta inflamatoria **(Figura 6)**.

Siguiendo este razonamiento, encontramos que miR-335 era el único miRNA cuya expresión era significativamente mayor en el estado inactivo o indiferenciado de las hMSCs en comparación con hMSCs parcialmente diferenciadas a hueso y grasa, y células mesenquimales totalmente diferenciadas, como los fibroblastos.

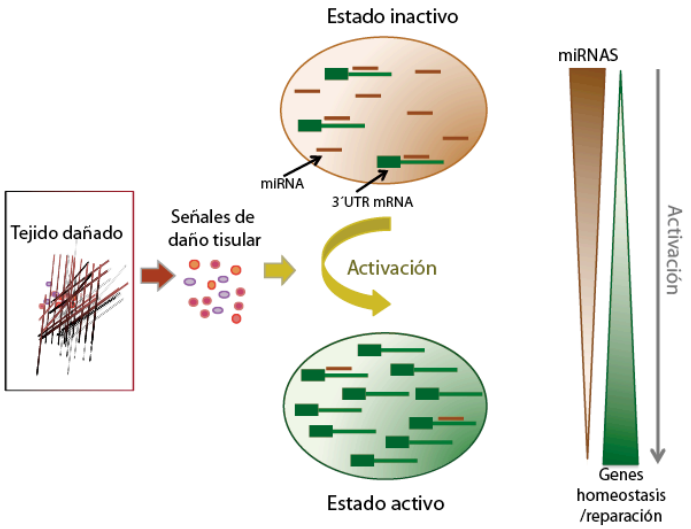

Figura 6. Hipótesis del mecanismo de activación de las células madre. Algunos miRNAs están presentes en el estado inactivo/indiferenciado de las hMSCs reprimiendo la expresión de numerosos genes. Tras la estimulación mediante señales provenientes de tejidos dañados, las hMSCs se activan, disminuyendo los niveles de ciertos miRNAS y permitiendo la expresión de dichos genes implicados en distintos programas de biológicos. Adaptado de Cheung y Rando[216].

Por tanto, miR-335 muy probablemente contribuiría al mantenimiento de las hMSCs en un estado de inactivación (*resting* o quiescente), siendo necesaria su regulación a la baja para adquirir el estado activo de las mismas en respuesta a distintos estímulos.

Nuestros resultados demuestran que la regulación de la expresión de miR-335 está íntimamente relacionada con las señales encargadas de regular la biología de las hMSCs. Por un lado, el tratamiento de las hMSCs con Wnt3a, un activador de la ruta de Wnt/β-catenina, aumentó la expresión de miR-335, por otro, el tratamiento con DKK1, un inhibidor de Wnt, promovió su disminución. El papel de Wnt3a en la biología de las hMSCs no parece estar completamente esclarecido. Unos estudios sugieren que la señalización de Wnt promueve la proliferación y mantiene el estado indiferenciado de las hMSCs[217, 218], mientras que otro estudio afirma que Wnt3a es capaz de promover proliferación en hMSCs, pero que a grandes dosis es capaz de inhibirla[219]. Nuestros resultados apoyan un papel inhibidor de Wnt en la diferenciación de las hMSCs, y ponen de manifiesto la importancia del papel de miR-335 en la regulación de la biología de estas células mediada por Wnt/β-catenina.

Por otra parte, se ha demostrado que otros muchos estímulos promueven la adquisición de un fenotipo activado de las hMSCs. Así, por ejemplo, el tratamiento con IFNγ es suficiente para que las MSCs adquieran un fenotipo inmunorregulador[65], CXCL12 contribuye a la migración de las hMSCs a los tejidos dañados[26] y el uso de factores definidos induce la osteogénesis y adipogénesis de las hMSCs[220]. El tratamiento con cada uno de estos estímulos específicos provocó la disminución de la expresión de miR-335 en las hMSCs, lo cual sugiere que la regulación a la baja de miR-335 sería parte del mecanismo de respuesta rápida frente a todos estos estímulos de activación, permitiendo la salida del estado inactivo (*resting*) de las hMSCs, mantenido por niveles relativamente mayores de este miRNA.

Recientemente, se ha determinado que varios genes encargados de controlar el crecimiento, están también implicados en la biología de las células madre adultas. Estos genes están sujetos al fenómeno de "*imprinting*", un mecanismo genético por el que ciertos genes, que únicamente representan el 1% de todos los genes humanos y de ratón[221], se expresan de forma monoalélica, dependiendo del origen parental. En paralelo a nuestro trabajo, otros autores han descrito que la expresión de estos genes, entre los cuales se encuentra *Mest*, el gen hospedador de miR-335, es muy alta en células madre adultas, y disminuye tras recibir estímulos para su activación/diferenciación, al igual que observamos con la regulación de miR-335 en las hMSCs. Además, estos genes se expresan a bajo nivel en células somáticas diferenciadas, en comparación con las células madre adultas de las

que derivan[222]. Nuestros resultados corroboran dicha hipótesis, puesto que encontramos una expresión relativa elevada de miR-335, así como de MEST, en diferentes fuentes de hMSCs en comparación con fibroblastos dérmicos, hecho que también fue descrito por otros autores durante el desarrollo de nuestra investigación[223].

En conclusión, nuestros resultados demuestran que miR-335 (y probablemente también su gen hospedador, MEST) contribuye de forma crítica a la activación de las hMSCs , proceso indispensable para llevar a cabo los procesos de reparación tisular.

2. La sobreexpresión de miR-335 inhibe las capacidades reparadoras de las hMSCs.

La sobreexpresión forzada de miR-335 en hMSCs (hMSCs-335) mantiene a las hMSCs en un estado de inactivación, capaz de reprimir sus propiedades reparativas: proliferación, diferenciación y migración.

Proliferación.

La sobreexpresión de miR-335 redujo la capacidad de proliferación de las hMSCs; este mismo efecto ha sido también observado por el grupo de Benetti en mESC y en una línea celular de osteosarcoma humano[224, 225]. Estos estudios identificaron pRB como diana directa de miR-335, tanto en humano como en ratón. La hipótesis propuesta es que la inhibición de pRB promueve una ventaja en el crecimiento de las células que es compensada con el incremento de p53, resultando finalmente en la drástica disminución de la capacidad de proliferación. En nuestro estudio, sin embargo, no observamos ninguna alteración significativa del ciclo celular y tampoco se observó un incremento de los procesos de apoptosis o necrosis. Estos resultados, por tanto, no apoyan un papel central de p53/p21 en la inhibición de la proliferación inducida por miR-335 en hMSCs.

Diferenciación.

La sobreexpresión de miR-335 disminuyó drásticamente la capacidad de diferenciación de las hMSCs a hueso, grasa y cartílago.

Diferenciación ósea: Nuestros resultados demuestran inequívocamente que las hMSCs-335 poseen una menor capacidad de diferenciación ósea, tanto *in vitro* como *in vivo*, en comparación con las células control. Además, hemos demostrado que RUNX2, un factor de transcripción esencial para la diferenciación osteogénica, es una diana directa de

miR-335, lo cual explicaría, al menos parcialmente la casi nula capacidad osteogénica observada en las hMSCs-335.

Sin embargo, otros autores han descrito recientemente que la sobreexpresión de miR-335 es capaz de incrementar la diferenciación en MSCs de ratón (mMSC), inhibiendo la expresión de su gen diana DKK1, un antagonista de Wnt[226]. Este aumento en la señalización vía Wnt sería capaz de promover la expresión de genes relacionados con la osteogénesis, como Runx2, *Ibsp y Bglap*.

Como comentamos con anterioridad, en lo que concierne al efecto mediado por la señalización de Wnt en MSCs existen discrepancias. En concreto, los efectos *in vitro* mediados por la ruta de Wnt en la diferenciación osteogénica en MSC son muy controvertidos; por un lado se ha propuesto que estimula la diferenciación de MSCs murinas [227, 228], mientras que en MSCs humanas se ha descrito que tiene un efecto tanto estimulatorio[229] como inhibitorio[217, 230]. Todos estos datos sugieren que pueden existir diferencias críticas en el papel que juega la ruta de Wnt en MSC de diferentes especies y, probablemente, entre diferentes linajes celulares.

Sin embargo, cabe destacar que entre las dianas directas de miR-335 descritas en humanos por otros estudios se encuentra ROCK1[231], una quinasa que promueve la diferenciación osteogénica de las hMSCs[232], lo cual estaría en concordancia con nuestra identificación de RUNX2 como diana directa de este miRNA, y por tanto con que la sobreexpresión de miR-335 inhiba la diferenciación osteogénica en MSCs de humanos, tal y como demuestran nuestros resultados.

Diferenciación a tejido adiposo: Nuestros estudios muestran que la sobreexpresión de miR-335 inhibe la diferenciación de las hMSCs a tejido adiposo, y que la expresión de miR-335 baja al comienzo del proceso de diferenciación. Por el contrario, otros estudios relacionan positivamente la expresión de miR-335 con el metabolismo lipídico[233]. Nakanishi y cols. observaron que la grasa proveniente de ratones obesos, ob/ob, contiene una alta cantidad de miR-335 en comparación con ratones control. Además, este grupo demostró el aumento de la expresión de miR-335 durante la diferenciación adipogénica de una línea de fibroblastos embrionarios de ratón. Estos datos, obtenidos en ratón, de nuevo pondrían de manifiesto que los mecanismos implicados en ratón y humano podrían ser opuestos en lo que a diferenciación se refiere.

Diferenciación a cartílago: Para completar el estudio del papel de miR-335 en la capacidad multipotente de las hMSCs, usamos un modelo *in vivo* de diferenciación osteocondral mediante el uso de cámaras de difusión. En este estudio, fuimos capaces de observar que la sobreexpresión de miR-335 impidió la diferenciación a hueso y cartílago *in*

vivo. Una vez más, estudios realizados en mMSCs observaron el incremento de la expresión de miR-335 durante su diferenciación a condrocitos[234], teniendo a Daam1 y ROCK1 como dianas principales, las cuales regulan negativamente SOX9, principal regulador de la condrogénesis [235]. En este estudio, además, se describe que miR-335 disminuye los niveles de DKK1 durante la diferenciación condrogénica de mMSCs.

Por el contrario, los datos previos disponibles en humanos indican que los niveles de DKK1 aumentan durante la diferenciación a condrocitos[236]; incluso la adición exógena de DKK1 es capaz de aumentar la condrogénesis[237, 238], incrementando la expresión de SOX9. Por tanto, sería de esperar que durante la diferenciación a cartílago se produjese una disminución de miR-335 a consecuencia del aumento de DKK1, al contrario que lo observado en ratón. De hecho, nuestros resultados demuestran que DKK1 disminuye los niveles de miR-335 en las hMSCs.

Tomados en conjunto los resultados de nuestra investigación y los publicados por otros autores, se puede indicar que en humano y ratón existen importantes discrepancias en la regulación de los mecanismos implicados en la diferenciación de MSCs. En concreto, las rutas de Wnt/beta catenina y DKK1 parecen tener papeles completamente opuestos. Estos datos sugieren que el modelo de ratón podría no ser el más adecuado para el estudio de algunas funciones de las hMSCs, así como de otros procesos muy relevantes en salud humana, como por ejemplo los relacionados con la obesidad.

Inmunorregulación.

El papel de miR-335 en la actividad inmunorreguladora de las hMSCs fue analizado usando un modelo murino de endotoxemia, que produce una respuesta inflamatoria sistémica aguda que conduce a la muerte en pocos días. La endotoxemia mediada por LPS activa las células del sistema inmune innato[239], e induce la expresión de mediadores proinflamatorios como TNFα e IL6[240]. Estudios previos muestran que las hMSCs son capaces de disminuir la inflamación, disminuyendo los niveles de citoquinas proinflamatorias[241] y prolongando la supervivencia. En nuestro modelo, el tratamiento con hMSCs sobreexpresando miR-335 disminuyó severamente la capacidad protectora de las células, eliminando su potencial terapéutico en esta patología.

En cuanto a los mecanismos por los que miR-335 inhibe el efecto antiinflamatorio, observamos que, aunque en menor medida que las células control, las hMSCs-335 fueron capaces de inhibir significativamente la proliferación de linfocitos. Además, las hMSCs-335 reprogramaron el fenotipo de los macrófagos al igual que las hMSCs-control, de un

estado proinflamatorio “M1” (caracterizado por la secreción de citoquinas proinflamatorias TNFα e IL6) a un estado antiinflamatorio “M2” (caracterizado por la secreción de citoquina IL10 antiinflamatoria)[62, 242]. Es decir, las hMSCs-335 mostraron claramente una menor capacidad antiinflamatoria *in vivo*, pero ello no parece ser consecuencia de una incapacidad intrínseca de regular a las células del sistema inmune, como linfocitos y macrófagos.

Migración.

Uno de los procesos más importantes para comenzar el proceso de inmunorregulación es la movilización de las hMSCs al lugar de la inflamación, proceso conocido como migración celular. Algunos trabajos apuntan incluso a que la migración es un mecanismo fundamental para modular la respuesta inflamatoria durante la sepsis[243].

Nuestros resultados demuestran que la sobreexpresión de miR-335 inhibe la capacidad de migración de las hMSCs. Este hallazgo no resulta sorprendente si tenemos en cuenta el efecto inhibitorio de miR-335 sobre la capacidad migratoria de varios tipos de células tumorales que ya había sido descrito previamente, lo que ha llevado a considerar a este miRNA como un potente supresor de metástasis[204]. En estos trabajos, se demostró que miR-335 inhibe la migración e invasión de células metastásicas de cáncer de mama mediante la regulación de genes diana como SOX4 y TNC.

Nuestros resultados demuestran que miR-335 es capaz de suprimir la capacidad de migración en respuesta a una amplia batería de factores específicos. Además, varios estudios han descrito recientemente que los macrófagos, unas de las primeras células activadas en la respuesta inmune, son los principales encargados de mediar la migración de las hMSCs al lugar de inflamación[50]. Usando medio condicionado proveniente de macrófagos activados (CM-Mϕ), observamos que la sobreexpresión de miR-335 redujo de manera drástica la migración de las hMSCs.

Por otro lado, la sobreexpresión de miR-335 provocó la disminución en la expresión del receptor de CXCL12, uno de los principales factores que median la migración de hMSCs[26]. Este hallazgo, sin embargo, no explicaría por sí solo la inhibición de la migración observada ante la gran variedad de estímulos usados.

Nuestros resultados parecen indicar que, aunque las hMSCs-335 conservan casi por completo toda su capacidad de modular a las células del sistema inmune, poseen algún defecto importante en su capacidad migratoria, lo cual se traduciría en una disminución del

número de células capaces de alcanzar el lugar de inflamación, reduciendo el efecto antiinflamatorio global de las hMSCs *in vivo*.

3. La expresión de miR-335 se correlaciona con el envejecimiento en las hMSCs.

En nuestro estudio encontramos que la expresión de miR-335 aumenta durante el cultivo *ex vivo* de las hMSCs y, además, sus niveles muestran una correlación directa con la edad de los donantes de las que son aisladas. Sin embargo, tras inducir senescencia mediante radiación gamma no observamos un aumento significativo de este miRNA. Se sabe que la senescencia inducida por irradiación usa mecanismos distintos a la senescencia replicativa; así, por ejemplo, se ha observado en fibroblastos que los niveles de p16 no aumentan tras la radiación, mientras sí lo hacen durante la senescencia replicativa[244], aunque el fenotipo senescente final sea el mismo. Es decir, probablemente la senescencia inducida por irradiación está mediada por otras vías distintas a miR-335.

Por otro lado, cuando las hMSCs fueron sometidas a estímulos capaces de frenar el proceso de senescencia, como la sobreexpresión exógena de TERT y la reducción del oxígeno en cultivo[132, 245], los niveles de miR-335 disminuyeron. Además, el tratamiento con estímulos que promueven su activación, como diversos factores de crecimiento, fueron también capaces de promover la disminución de la expresión de miR-335.

Estos resultados nos llevan a pensar que, si bien los niveles basales de miR-335 encontrados en hMSCs de diferentes fuentes deben disminuir para que la célula pueda activarse, el proceso de envejecimiento promovería un aumento de miR-335 por encima de cierto umbral, favoreciendo que la célula entre en un estado de inactividad, o senescencia. Otros estudios también han demostrado niveles elevados de MEST en hMSCs aisladas de médula ósea provenientes de donantes de edad avanzada, en comparación con los niveles encontrados en hMSCs de donantes jóvenes[246]. Asimismo, otro estudio muestra que la expresión, entre otros miRNAs, de miR-335, aumenta en hMSCs de tejido adiposo proveniente de donantes de edad avanzada[199]. Por otro lado, otros estudios realizados en ratón también correlacionan los niveles de miR-335 con el grado de senescencia; así, se observa una mayor expresión de miR-335 en riñón[247], en corazón [248] y en células endoteliales[249] en ratones de avanzada edad en comparación con ratones más jóvenes.

Por tanto, parece claro que el proceso del envejecimiento regula al alza los niveles de este miRNA en MSCs y otros tipos celulares tanto en ratón como en humano.

4. La sobreexpresión mantenida de miR-335 produce la adquisición de un fenotipo senescente en hMSCs.

Todos los numerosos cambios que hemos observado en las hMSCs como consecuencia de la sobreexpresión de miR-335 indican que los altos niveles de dicho miRNA inducen en estas células la adquisición de un fenotipo senescente. Una observación similar ha sido descrita en células mesangiales de rata sobreexpresando miR-335[247].

Los cultivos de hMSCs-335 presentaron un mayor porcentaje de células positivas para beta-galactosidasa, y una menor capacidad de proliferación, que puede ser atribuida principalmente al aumento de los niveles de p16 observados[250]. Por el contrario, los niveles de p53 y p21 fueron ligeramente más bajos en hMSCs-335. A este respecto, varios estudios han observado que la activación de p21 y p53 en células senescentes es transitoria, y los niveles de ambos disminuyen una vez se ha llegado a establecer el arresto proliferativo, mientras que los niveles de p16 continúan aumentando[244, 251], lo cual explicaría los menores niveles de p21 y p53 encontrados. Además, las hMSCs-335 también mostraron un incremento de los niveles de CCND1, de forma similar a lo ya sido descrito en fibroblastos senescentes[244].

Estos hallazgos nos llevan a pensar que el arresto proliferativo encontrado en las hMSCs-335 sería mediado principalmente por el aumento exacerbado de p16. De hecho, ya había sido propuesto anteriormente que la inducción de p16, pero no p21, es responsable de la senescencia prematura en hMSCs[252]. Nuestros resultados corroborarían además otros estudios previos realizados por el grupo de Benetti en los cuales se describe que la sobreexpresión de miR-335 disminuye la proliferación celular a través de la represión de su gen diana pRB[224, 225]. Como ya indicamos anteriormente, estos autores atribuyen el incremento en la expresión de p53 a un mecanismo compensatorio para reprimir la ventaja proliferativa que produce la inhibición de pRB. Sin embargo, las células utilizadas por dicho grupo, U2OS, son una línea celular de osteosarcoma, sin expresión de p16 (debido a la hipermetilación de sus promotores), lo cual sugiere que, en células no tumorales, donde la expresión de p16 no se encuentra alterada, miR-335 podría actuar preferentemente a través de p16 en vez de con p53[253]. Este fenómeno podría explicar el aumento en la cantidad de proteína p16 que observamos en hMSCs-335, así como la propia reducción en su capacidad de proliferación. De hecho, ya en 1994 se demostró en células en cultivo que pRB reprime la actividad transcripcional del promotor del gen de p16[254] . Unos años más tarde se encontró que la expresión de p16 disminuye la expresión de pRB en células humanas[255], y recientemente se ha encontrado que p16 es además capaz de disminuir la fosforilación de pRB, produciendo un estado de arresto proliferativo[256]. Por tanto, la

elevada expresión de p16 observada en las hMSCs-335 podría explicarse como consecuencia de la disminución de la expresión de pRB mediada por miR-335, y a su vez p16 podría disminuir los niveles de pRB y de su forma fosforilada, reprimiendo como consecuencia la proliferación de las hMSCs.

Además, las hMSCs-335 presentaron un tamaño mucho más grande en comparación con las hMSCs-control, así como una morfología irregular en su citoplasma y núcleos, propia de células senescentes[136-138]. En consonancia, la sobreexpresión de miR-335 disminuye la expresión de lamina B1, la cual contribuye a mantener la estructura del núcleo, lo cual explicaría la forma irregular de los núcleos encontrados; la reducción de la expresión de lamina B1 ha sido correlacionada con senescencia[257]. Otra característica que indica senescencia celular es que las hMSCs-335 poseían un patrón de expresión de SCIN, EDN1, AKAP9 y CXCL12 similar al definido recientemente para hMSCs senescentes[258].

De forma adicional, encontramos en las hMSCs-335 una dramática disminución en los niveles de proteína SOD2, una enzima encargada de disminuir las especies reactivas de oxígeno (ROS), y que ha sido también relacionada con la adquisición del fenotipo senescente debida al aumento de los niveles de p16[259]. Nuestros resultados demuestran que en las hMSCs-335 no sólo aumenta la cantidad de ROS específicas de mitocondria, sino también los niveles globales de ROS. Este mismo resultado ha sido corroborado por otros autores, que han identificado SOD2 como una diana directa de miR-335[247].

En cuanto al fenotipo secretor, observamos que el aumento de los niveles de miR-335 promovió la secreción de un panel de moléculas similar al descrito previamente en células humanas y murinas, conocido como SASP[260]. Entre las numerosas moléculas secretadas por las hMSCs-335 en mayores niveles que las células control encontramos importantes cantidades de citoquinas proinflamatorias como IL8 e IL6, y de factores reguladores de la adhesión como VCAM1 y MCP1.

MCP1 promueve la migración de macrófagos[261] y basófilos, y tiene un papel central en varias enfermedades inflamatorias[262]. IL8 también está implicada en la quimioatracción de los neutrófilos[263]. Por otro lado, se conoce que VCAM1 promueve la adhesión de las células T[74]. Por tanto, estos componentes del secretoma de hMSCs-335 promoverían la atracción de distintos tipos de células del sistema inmune.

En cuanto a la función de IL6, ha sido descrito que sus niveles aumentan en pacientes de avanzada edad[143]; incluso varios estudios confirman que los niveles de IL6 en suero sirven de indicador de mortalidad en dichos pacientes[145-147]. Esto relacionaría la elevada expresión encontrada en hMSCs-335 con su implicación en senescencia y envejecimiento. Además, el aumento en los niveles de IL6 produce una moderada

inflamación crónica, en línea con la hipótesis de que parece haber una estrecha relación entre envejecimiento e inflamación, conocida como "*inflamaging*"[264]. En este mismo sentido, se ha confirmado que la exposición constante a IL6 inhibe la capacidad de los macrófagos de producir citoquinas antiinflamatorias, lo que les impide adquirir el fenotipo M2 (inmunomodulador)[265].

Por tanto, el patrón de moléculas secretado por las hMSCs-335 sugiere que altos niveles de miR-335 propiciarían probablemente el reclutamiento y proliferación de las células del sistema inmune a sus nichos por todo el organismo, contribuyendo al establecimiento de un estado de inflamación crónica que ya ha sido asociado a la senescencia de las hMSCs. De hecho, según otro estudio, las células madre procedentes de pacientes obesos tienen disminuida su capacidad de proliferación y potencial de diferenciación, y presentan un fenotipo senescente[266]. El grupo de Minamino analizó el tejido adiposo en ratón obeso (ob/ob), observando que este tejido presentaba signos de estrés oxidativo, envejecimiento e inflamación[267]. De ahí que los niveles de miR-335 encontrados en ratones obesos en comparación con ratones control[233], inicialmente asociados al metabolismo lipídico, probablemente sean debidos más bien a que la obesidad, al promover la senescencia, incremente los niveles de este miRNA en dichos tejidos.

Por otra parte, hemos analizado el SASP en hMSCs inducidas a senescencia por radiación gamma, y éste es muy similar al que encontramos en las hMSCs-335, aunque las cantidades secretadas fueron superiores en el primer caso. Aunque la senescencia de las hMSCs ha sido previamente caracterizada[268], y el conocimiento de que la senescencia produzca un fenotipo secretor característico data del año 2008[269], nuestro estudio es el primero en describir el SASP de las hMSCs senescentes. Entre las moléculas que forman parte del SASP, en hMSCs senescentes encontramos MCP1, IL8, IL6 y VCAM1. Al igual que ocurre con IL6, el incremento de los niveles de VCAM1 ha sido descrito en sujetos humanos de avanzada edad[270].

Por último, uno de los aspectos más relevantes de la senescencia de las hMSCs, corroborado tanto *in vitro* como *in vivo*, es que suprime la funcionalidad de estas células[152, 153, 171]. Como ya indicamos anteriormente, la sobreexpresión de miR-335 es capaz de inhibir todas las propiedades terapéuticas de las hMSCs, lo cual confirmaría, más aún si cabe, que la expresión de miR-335 promueve la senescencia de las hMSCs. Hay que decir, sin embargo, que, hasta el momento, no hemos logrado la regulación estable a la baja de miR-335 en las células que lo sobreexpresan de forma exógena, por lo que no podemos afirmar que la senescencia inducida por este miRNA sea, al igual que la senescencia replicativa, un proceso irreversible.

5. miR-335 inhibe las propiedades terapéuticas de las hMSCs a través de la represión del complejo AP-1.

Las hMSCs se mantienen "inactivas" cuando están en un estado de quiescencia (arresto reversible) o bien cuando alcanzan la senescencia (arresto irreversible). Por tanto, los mecanismos encargados de mantener a la célula de manera "no funcional" podrían estar estrechamente relacionados. De hecho, varios trabajos recientes establecen un nexo de unión entre quiescencia y senescencia; por un lado parece que las células más envejecidas posen un mayor grado de quiescencia[271] y por otro parece que las células que permanecen durante largos periodos de forma quiescente se vuelven senescentes[256].

El análisis con microarrays del patrón de expresión diferencial en células hMSCs-335 nos permitió observar que la mayoría de los miembros del complejo AP-1 (incluyendo MAF, ATF3, JUN, JUNB, FOS y FOSB) se encontraban significativamente expresados a la baja en comparación con las hMSCs-control. Estos resultados fueron confirmados mediante RT-qPCR. AP-1 es un factor de transcripción cuya activación, mediada por las MAPKs, requiere la fosforilación de sus componentes, además está implicado en la regulación de un sin fin de procesos biológicos fundamentales como proliferación, apoptosis y diferenciación, entre otros[272]. La implicación de la regulación del complejo AP-1 en el mantenimiento del estado inactivo en las células ha sido bien documentada. Así, por ejemplo, se ha descrito que los componentes fosforilados de AP-1 se encuentran en menores cantidades en células senescentes[199], y que la inhibición de las MAPKs mantiene el estado quiescente, actuando como un interruptor para su activación[273]. Por otro lado, la inhibición de JNK (quinasa implicada en la ruta de activación de AP-1) induce el arresto permanente del ciclo celular, lo que indica que los niveles basales de JNK son esenciales para prevenir la senescencia prematura[274]. También se ha demostrado que la inhibición de MKK7, un activador de JNK, promueve la senescencia prematura[275]. Por tanto, la disminución en la expresión de los componentes de AP-1 por parte de miR-335 podría contribuir a mantener a las hMSCs en un estado de represión que probablemente explicaría la falta de funcionalidad observada en las hMSCs que sobreexpresan este miRNA.

Inmunorregulación y migración celular.

Recientemente ha sido descrito que la activación del complejo AP-1 posiblemente sea uno de los principales mecanismos que controlan la migración de las hMSCs al lugar de daño. En ese mismo trabajo se propone que son los macrófagos activados los que guían la migración de las hMSCs, mediante la secreción de numerosos factores[50].

El tratamiento de las hMSCs-335 con el medio condicionado de macrófagos activos (CM-Mϕ) puso de manifiesto la represión de la activación de los componentes del complejo AP-1 FOS y JUN, así como de sus variantes fosforiladas. PRKD1, una serina/treonina quinasa, promueve la activación de las MAPKs[276, 277], las cuales, entre otras funciones, promueven la activación del complejo AP-1. La inhibición de la activación de PRKD1 por parte de miR-335 y la consiguiente inhibición de AP-1, confirma otros estudios que demuestran que uno de los principales mecanismos mediados por miR-335 para inhibir la invasión de células metastásicas es la represión de MAPK1[231]. Este último trabajo, además, señala que AP-1 es capaz de activar MMP-9, una metaloproteinasa encargada de mediar la invasión a través de endotelio. Por tanto, la represión de la activación de AP-1 podría bloquear la migración e invasión de las hMSCs al lugar del daño, lo cual no sólo reduciría su efecto antiinflamatorio, sino su efecto reparativo global.

Numerosos trabajos apuntan a que el LPS y el TNFα se unen a los receptores TLR4 y TNFR1, respectivamente, aumentando la expresión de PTGS2, y por tanto la producción de PEG2, un importante factor inmunorregulador producido por las hMSCs. El tratamiento de las hMSCs con CM-ϕ (el cual contiene elevadas cantidades de TNFα) produjo, como era de esperar, un importante incremento de la expresión de PTGS2, incremento que resultó drásticamente inhibido en las hMSCs-335. Sin embargo, a pesar de esta importante disminución de los niveles de PTGS2, observamos que el incremento de IL10 en macrófagos en contacto con hMSCs-335 (indicativo de un fenotipo inmunomodulador M2) fue muy similar al encontrado en el cocultivo con hMSCs-control. Estos resultados, contrariamente a lo descrito por otros autores[62], no parecen soportar que PEG2, producido por PTGS2, juegue un papel importante en la modulación del fenotipo de los macrófagos.

Diferenciación.

Además de la migración, AP-1 regula la síntesis de DNA, la progresión de ciclo celular[278], la diferenciación osteoblástica y la condrogénesis[279]. En este sentido, los macrófagos parecen regular la diferenciación osteogénica de las hMSCs[52, 280] mediante la oncostatina M (OSM), la cual activa diversas cascadas, entre las que se encuentra la de las MAPKs. Por tanto, el tratamiento previo con el medio CM-ϕ podría también estar promoviendo no sólo la migración de las hMSCs, sino también su diferenciación temprana. La represión de AP-1 encontrada en las hMSCs-335, por tanto, contribuye muy probablemente mediante este mecanismo a la baja capacidad osteogénica de las hMSCs[281].

Está bien documentado que el tratamiento de las hMSCs con BMP2, un miembro de la familia de factores de crecimiento, induce diferenciación ósea y condrogénica en las

MSCs[282, 283]. Varios estudios muestran que BMP2 produce la diferenciación osteoblástica vía PRKD1/MAPK[284, 285]. Nuestros resultados indican que la sobreexpresión de miR-335 fue capaz de inhibir la activación del complejo AP-1 frente al tratamiento con BMP2, lo cual sugiere un papel clave de este factor de transcripción en el control de la expresión génica durante los estadios tempranos de la diferenciación osteoblástica y condrogénica, y también explicaría, al menos en parte, la pérdida de la capacidad de diferenciación osteocondral observada en las hMSCs-335.

Todos nuestros resultados, tomados en su conjunto, sugieren que los niveles basales de miR-335 mantendrían a las hMSCs en un estado quiescente, permitiendo a las células responder a determinados estímulos mediante su regulación a la baja (mediada por las rutas activadas por dichos estímulos). Por el contrario, durante el proceso de senescencia o envejecimiento los niveles de miR-335 aumentarían por encima de un determinado umbral que podría derivar en un arresto permanente, mediante, entre otros mecanismos, la supresión crónica de AP-1, la cual bloquearía la capacidad de respuesta de las hMSCs frente a prácticamente cualquier estímulo y, en definitiva, disminuiría drásticamente la funcionalidad de estas células.

6. La senescencia inhibe la capacidad migratoria de las hMSCs a través de la inactivación del complejo AP-1.

De forma análoga a los resultados obtenidos en hMSCs-335, en las hMSCs inducidas a senescencia por radiación o replicación celular prolongada observamos una drástica reducción de su capacidad migratoria en comparación con las hMSCs presenescentes, usando como estímulo CM-Mϕ. Guiándonos por los resultados encontrados en las hMSCs-335, medimos los componentes del complejo AP-1 tras la estimulación con CM-Mϕ y encontramos que, como era de esperar, la activación del complejo AP-1 está muy reducida en células senescentes en comparación con las presenescentes. Además, tras someter a las hMSCs presenescentes y senescentes a un proceso de "wound healing" observamos que las primeras son capaces de incrementar drásticamente los niveles de FOS 90 minutos después de producirse la herida, mientras que las segundas continuaron reprimiendo la expresión de FOS y por consiguiente la activación de AP-1. Las consecuencias de esta inhibición podrían explicar en gran medida la reducida funcionalidad de las hMSCs senescentes, de forma similar a lo observado en las hMSCs-335.

Visión global.

El sistema miR-335/AP-1 actúa como un interruptor molecular capaz de regular las principales respuestas biológicas de las hMSCs.

En condiciones basales, la expresión de miR-335 mantiene a las hMSCs en un estado quiescente o inactivo. Cuando las hMSCs reciben las señales adecuadas, la expresión de miR-335 disminuye, permitiendo la activación de la célula, para que ésta pueda llevar a cabo sus propiedades terapéuticas.

Por otro lado, el envejecimiento y la senescencia replicativa producen un incremento drástico de los niveles de miR-335 que, al igual que la sobreexpresión exógena de este miRNA, induce una fuerte represión del complejo AP-1. La inactivación de AP-1 inhibe la funcionalidad de las hMSCs, impidiéndolas llevar a cabo eficazmente procesos como proliferación, migración, diferenciación e inmunorregulación en respuesta a señales de daño tisular (**Figura 7).**

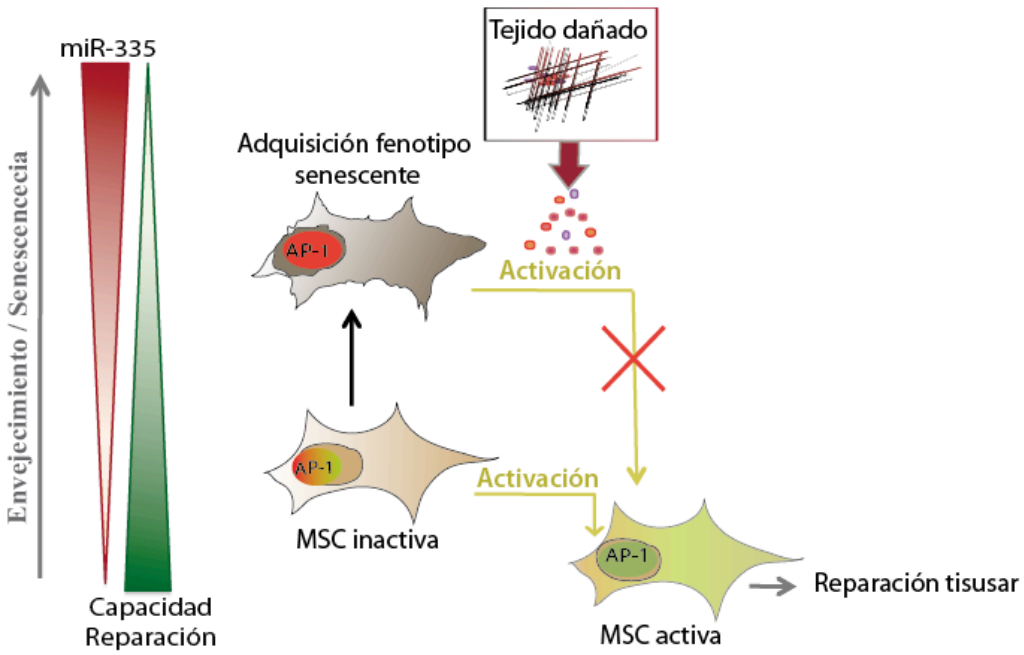

Figura 7. Modelo de la función de miR-335 en la regulación de la actividad de las hMSCs. La expresión basal de miR-335 mantiene a las hMSCs en un estado inactivo. Tras su activación, mediada por señales provenientes de tejidos dañados, la expresión de miR-335 disminuye, permitiendo la expresión de los genes que permiten a estas células llevar a cabo la reparación tisular. Por otra parte, el proceso de envejecimiento promueve un aumento importante de miR-335, el cual induce la aparición del fenotipo senescente, que va acompañado de la represión del complejo AP-1, la cual reduce drásticamente la funcionalidad de las hMSCs senescentes.

CONCLUSIONES

1. **La disminución de la expresión de miR-335 es un mecanismo esencial para la activación de las hMSCs.**

2. **miR-335 y su gen hospedador MEST están regulados por señales importantes en el control de la biología de las hMSCs.**

3. **Existe, al menos en las hMSCs, una correlación directa entre el nivel de expresión de miR-335 y el envejecimiento.**

4. **La expresión forzada de miR-335 en hMSCs limita drásticamente sus diversas capacidades terapéuticas.**

5. **La expresión mantenida de miR-335 en hMSCs promueve el desarrollo de un fenotipo senescente.**

6. **miR-335 inhibe las propiedades terapéuticas de las hMSCs a través de la represión del complejo AP-1.**

7. **Los mecanismos implicados en la pérdida de funcionalidad de las hMSCs con altos niveles de miR-335 son similares a los encontrados en hMSCs que han alcanzado la senescencia por replicación o por radiación.**

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